

STRUCTURE, EXPRESSION AND FUNCTION OF THE DROSOPHILA
SEX-DETERMINING GENE «TRANSFORMER»

INAUGURAL-DISSERTATION

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der
PHILOSOPHISCHEN FAKULTÄT II
der
UNIVERSITÄT ZÜRICH

von
IRMGARD IRMINGER-FINGER
aus
Regensburg/ZH

Begutachtet von
Herrn Prof. Dr. Rolf Nöthiger, Zürich
und
Herrn Prof. Dr. Martin Billeter, Zürich

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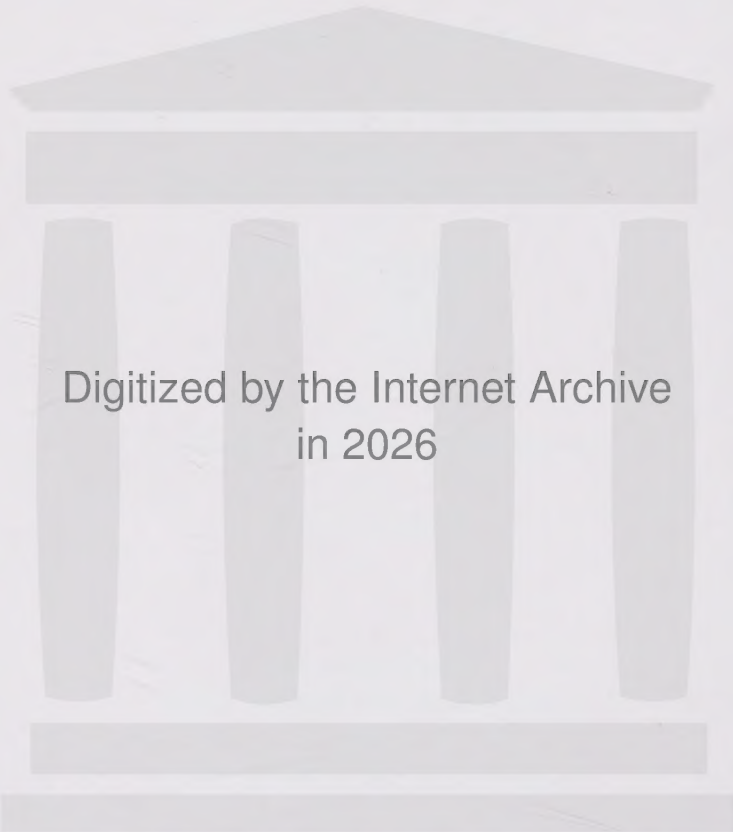
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ZUSAMMENFASSUNG

Das primäre Signal für die Geschlechtsbestimmung bei *Drosophila* ist das Verhältnis von X-Chromosomen zu Autosomensätzen. Einige wenige regulatorische Gene übermitteln dieses Signal zu den Geschlechtsdifferenzierungsgenen. Eine rezessive Mutation in einem der regulatorischen Gene, *transformer* (*tra*), bewirkt die Transformation von chromosomalen Weibchen zu phänotypischen Männchen. Diese Mutation zeigt jedoch keinen Effekt in XY Tieren. Die Expression des *tra* Gens ist abhängig von der Aktivität des übergeordneten Kontrollgens *Sex-lethal* (*Sxl*). Das *tra* Gen selbst ist an der Regulation von *doublesex* (*dxx*) beteiligt, dem letzten Gen in der regulatorischen Kaskade.

Unsere Gruppe hat das *tra* Gen durch "Microdissection" und "Chromosome-Walking" kloniert. Durch das Kartieren von chromosomalen Bruchpunkten und DNA-Aberrationen von spontanen Mutationen und durch Keimbahntransformation mittels P-Transformationsvektoren konnte nachgewiesen werden, dass die Funktion des *tra* Gens innerhalb eines DNA-Segments von 2kb lokalisiert ist, welches in adulten Männchen und Weibchen unterschiedlich transkribiert oder prozessiert wird.

In der vorliegenden Dissertation wird gezeigt, dass das funktionelle, weibchenspezifische *transformer* Transkript, welches nicht grösser als 1kb ist, in Larven- und Puppenstadien und in adulten Weibchen exprimiert wird. Von der Sequenz des genomischen DNA-Abschnittes konnten wir ein mögliches Protein von 142 Aminosäuren Länge ableiten. Die Aminosäuresequenz dieses Proteins weist keine Homologie zu anderen, bekannten Proteinen auf. Wir diskutieren unsere Resultate im Zusammenhang mit der entwicklungsbiologischen Funktion des *tra* Gens und die Möglichkeit, dass dieser Locus in die posttranskriptionelle Regula-

tion des Gens *dsx* eingreifen könnte, dessen Genprodukte für die korrekte Expression entweder der weiblichen oder der männlichen Differenzierungsgene notwendig sind.

SUMMARY

Sex determination in *Drosophila* is implemented by the ratio of X-chromosomes to sets of autosomes. A cascade of regulatory genes mediates this chromosomal signal down to the sex-differentiation genes. Recessive mutations of one of the regulatory genes, *transformer* (*tra*), transform chromosomal females (XX) into phenotypic males, but have no effect in XY flies. The expression of the *tra* functions depends on the differential activity of *Sex-lethal* (*Sxl*), the superior control gene. The *tra* gene in turn is involved in the regulation of *double-sex* (*dsx*), the last gene in the cascade.

Our group has cloned the *tra* gene by microdissection and chromosome walking. By mapping mutant lesions and by P-mediated transformation it could be shown that the *tra* function is harboured in a subsegment of 2kb that is differentially transcribed or processed in females and males.

In this paper we present evidence that the functional female-specific mRNA, which is expressed in larvae, pupae and in adult females, is not larger than 1kb and potentially codes for a protein of 142 aminoacids. The putative aminoacid sequence has no homology to other known proteins. We relate our findings to the developmental action of *tra* and speculate that the locus may be involved in the posttranscriptional regulation of *dsx*, whose products are known to be essential for the correct expression of either the male or the female set of sex-differentiation genes.



1. INTRODUCTION

The existence, origin and development of sexual polarity is one of the most fascinating and still unsettled problems of biology and sociological science as well.

Although the primary chromosomal sex-determining mechanism has been described in many species, in only three organisms do we begin to understand how this chromosomal signal is transformed into the concrete sexual phenotype: The yeast, *Saccharomyces cerevisiae* (Nasmyth, 1982), the nematode *Caenorhabditis elegans* (Hodgkin, 1985), and the fruit fly *Drosophila melanogaster* (Baker and Belote, 1983; Nöthiger and Steinmann-Zwicky, 1985). All three organisms use a short cascade of regulatory genes to transmit the initial signal down to the sex-differentiation genes.

Sex determination in the *Drosophila* embryo is established by its ratio of X-chromosomes to sets of autosomes (Bridges, 1921; Steinmann-Zwicky and Nöthiger, 1985a). The Y-chromosome is irrelevant for sex determination. Two or more X-chromosomes with two sets of autosomes lead to female development; one X and two or more sets of autosomes lead to male development; XX flies with three sets of autosomes develop an intersexual phenotype (Bridges, 1921; 1925).

Recent experiments suggest that although cells carry their sex-determining signal (the X:A ratio) with them throughout development, they seem to read this signal only early in development, long before most overt aspects of sexual differentiation occur (Sanchez and Nöthiger, 1983; Cline, 1985). Thus for *Drosophila*, the term sex determination seems to apply not only in its general sense, but also in the more restricted sense of developmental commitments maintained by divid-

ing cells. The classical paradigm for "determination" in this latter sense involves the process by which the precursor cells of the adult *Drosophila* integument acquire and maintain their segmental and subsegmental identities (Morata and Lawrence, 1977; Gehring, 1987).

A small number of genes has been identified that are involved in the transmission of the chromosomal signal, *Sex-lethal (Sxl)* (Cline, 1978), *daughterless (da)* (Cline, 1976; 1983), *transformer (tra)*, *transformer-2 (tra-2)*, *intersex (ix)* and *doublesex (dsx)* (Baker and Ridge, 1980). The differential activities of these genes are required for initiation, maintenance and expression of either the female or the male sexual pathway (for review, see Baker and Belote, 1983; Cline, 1985; Nöthiger and Steinmann-Zwicky, 1985). Mutations in all these genes lead to an inappropriate sexual phenotype compared with the respective chromosomal signal.

1.1. *Sxl* and the initiation of sexual development

The gene *Sxl*, on the X-chromosome, is known to control both sex determination and dosage compensation (Cline, 1978; Lucchesi and Skripsky, 1981; Steinmann-Zwicky and Nöthiger, 1985b) and to function in both the soma and the germ line (Schüpbach, 1985). It is activated in response to the chromosomal signal of two X-chromosomes and two sets of autosomes (Maine et al., 1985a). Its state of activity is irreversibly set at blastoderm stage (Sanchez and Nöthiger, 1983; Cline, 1984). Once this is achieved, it is the state of activity of *Sxl* that dictates either the female or the male sexual pathway. From now on, the X:A signal is no longer needed and the state of activity at the *Sxl* locus remains set.

Normal female development depends upon the continuous presence of one or more products of the *Sxl* gene. In contrast, these female-specific gene products must be absent for normal male development. Thus, loss-of-function alleles are lethal for females, but viable in males. Female lethality appears to be caused by the increase of X-chromosome gene expression in diplo-X individuals (Lucchesi and Skripsky, 1981; Baker and Belote, 1983; Lucchesi and Manning, 1987). These loss-of-function alleles cause transformation of genetically female cells towards a male phenotype, but early lethal effects of these alleles mask their sex-transforming effects (Cline, 1978; 1979; Sanchez and Nöthiger, 1982; Cline, 1984). Gain-of-function alleles express the gene's function regardless of the chromosomal sex, thus imposing the female pathway onto chromosomal males (Cline, 1978; 1979). These "constitutive" alleles lead to lethality in males as a result of decreased X-chromosome gene expression in haplo-X individuals. They do not have any deleterious effects on females.

1.2. The role of *dsx*, the last gene in the regulatory hierarchy

Sxl does not directly control the sexual differentiation genes, but uses at least three genes, namely *tra*, *tra-2* and *ix* to regulate *dsx*, the last gene in the hierarchy (Fig.1).

Genetically, the *dsx* locus can be subdivided into *dsx^f*, expressing a product F which specifies the female pathway, and *dsx^m*, expressing a product M which specifies the male pathway. Null alleles that abolish both functions produce an intersexual phenotype. XX; *tra/tra* and XX; *tra-2/tra-2* animals are transformed into males, whereas XX; *ix/ix* individuals develop an intersexual phenotype like mutants homozygous for *dsx*. This shows that absence of *tra* or *tra-2* leads to the

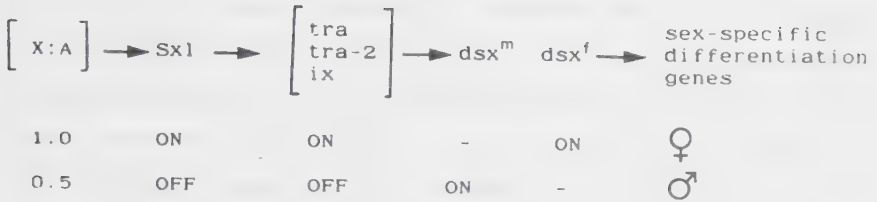


Fig.1. Outline of the genetic elements controlling the pathway of sexual differentiation in the soma.

expression of M, the product of dsx^m . This situation corresponds to the basic, male-determining state of the locus. The products of *tra* and *tra-2* prevent the expression of the M-function, but the product of *ix* is necessary for *dsx* to express the female function F (Nöthiger and Leuthold et al., 1987). With an X:A ratio of 0.5, the gene *Sxl* is not activated, *tra*, *tra-2* and *ix* remain silent, and the male function of *dsx*, M, is expressed. An X:A ratio of 1 sets *Sxl* to the female state of activity, *tra*, *tra-2* and *ix* are expressed, and the female function, F, is produced at the *dsx* locus.

1.3. Sex-determination is a cell-autonomous process

It was found by Sturtevant (1929) that genetic mosaics such as gynandromorphs consisting of XX and XO cells, develop female and male structures side by side, according to the sex-determining chromosomal signal of each cell. The same result was observed when the sex of a cell was changed by mitotic recombination in female larvae heterozygous for *Sxl*, *tra* or *tra-2*. The resulting homozygous clones formed male structures in a heterozygous female environment (Baker and Ridge, 1980; Sanchez and Nöthiger, 1982; Wieschaus and Nöthiger, 1982). These results indicate that the sex-determining genes act in a cell-autonomous manner.

1.4. Where and how do sex-determining genes act?

Sexual dimorphism is not limited to morphological structures such as genitalia, sex combs, cuticular pigmentation, but is also expressed in physiological and behavioral traits. This integral sexual phenotype depends on the activity of the sex-determining genes, ultimately on the presence of a *dsx* gene product, F in females and M in males. Homozygous mutations that affect the expression of *dsx^f* or *dsx^m*, such as *tra*, *tra-2* or *dsx^D*, affect all aspects of sex of an animal and transform XX individuals into phenotypic males, called pseudomales.

Two questions arise from these considerations:

-are the sex-determining products of *dsx*, M or F, expressed in all cells of an animal, or only in those tissues that display a sexual dimorphism?

-how can the same molecular signal, M or F, generate the variety of sexual characteristics, such as sex combs on the forelegs, behavior in the CNS, yolk protein synthesis in the fatbody, female or male genitalia, etc., etc.?

The first question can be answered by looking for sex-specific transcripts in different tissue of a larva or fly by "in situ" hybridization (Hafen et al., 1983; Fjose et al., 1985). No such data are available yet. Genetic experiments involving homeotic mutations and sexual characters, however, suggest that the sex-determining genes exert their function in all cells, even when we do not see a sexual dimorphism. They furthermore show that the local sexual phenotype results from the superposition and interaction of two genetic control systems represented by the sex-determining genes and the homeotic genes. Two examples shall illustrate this point:

i) The homeotic mutation *Polycomb* (*Pc*) or *extra sex combs* (*esc*) transform meso- and metathoracic legs into prothoracic legs (Lindsley and Grell, 1968). In XY males or in XX; *tra/tra* pseudomales, this transformation results in the appearance of sex combs not only on the first legs, but on all six legs. It seems safe to assume that *Pc* and *esc* do not regulate *tra* or *dsx*, and we may conclude that *dsx* expresses M not only in the forelegs, but also in the cells of the meso- and metathoracic legs. When these are homeotically transformed into forelegs, the presence of M and the absence of F lead to the formation of the conspicuous sex comb.

ii) *Abdominal B* (*Abd-B*), a dominant homeotic mutation in the Bithorax Complex (BX-C), transforms abdominal segments 5 to 8 (A5-A8) into abdominal segment 4 (A4) (Casanova et al., 1986). Since A4 does not display a sexual dimorphism, mutant males and females now look identical with respect to these segments that normally display a very pronounced sexual dimorphism (Bryant,

1978). Only when the genes of the BX-C are correctly expressed, do all the sexual differences appear that typically distinguish males from females in the last abdominal segments.

1.5. The sexually determined state is under permanent control of the regulatory genes

The products of the genes in the sex-determining regulatory hierarchy are required throughout development. This was shown by the production of homozygous clones of *Sxl^f*, *tra*, *tra-2* and *dsx*, induced by mitotic recombination in heterozygous females. The sexual phenotype of these clones is transformed from female to male when the genotype was changed even in the third larval and early pupal stage (Baker and Ridge, 1980; Epper and Nöthiger, 1982; Sanchez and Nöthiger, 1982).

The isolation of a temperature-sensitive mutation of the *tra-2* locus allowed Belote and Baker (1982) to analyze developmental aspects of sex determination in female and male individuals. Chromosomal females homozygous for *tra-2^{ts}* develop as females when raised at the permissive temperature of 16°C, but as males at the restrictive temperature of 29°C. By shifting such individuals from one temperature to the other at various times, it was found that functional products of *tra-2* are required throughout development for female sexual differentiation. Conversely, the absence of functional products is a prerequisite for male development (Epper and Bryant, 1983).

Few genes are known that are under the control of the sex-determining genes even in the adult stage. The best studied sex-specific terminal differentiation genes are the three yolk protein (YP) genes, YP1, YP2 and YP3 (for review see Postlethwait and Jowett, 1981). The expression of YP is restricted to the fatbody and follicle cells of adult females and is controlled by the sex-determining regulatory genes: chromosomal females that have been transformed into males as a result of homozygosity for a null mutation at either the *tra* or *tra-2* locus do not synthesize YP. Since XY; *dsx/dsx* intersexes produce YP, we conclude that the product of *dsx^m* is required to prevent YP synthesis in males (Bownes and Nöthiger, 1981). Temperature-shift experiments with homozygous XX; *tra-2^{ts}/tra-2^{ts}* animals showed that *tra-2* controls YP synthesis through the regulation of YP transcription or transcript stability (Belote et al., 1985a). When adult pseudomales reared at 29°C were shifted to 16°C, the YP genes started transcription already within 6 hour after the shift. Conversely, when adult females homozygous for *tra-2^{ts}* were shifted from 16°C to 29°C, the amount of YP and of YP RNA gradually decreased.

Another example of sex-specific differentiation which requires the expression of sex-determining regulatory genes during the adult period, is the production of female sex-pheromones in XX tissue (Tompkins, 1986). Also, the sex-specific expression of factors in the central nervous system (CNS) which control courtship behavior is dependent on the state of activity of *tra-2* throughout the adult period (cited in Baker et al., 1987). Homozygous *tra-2^{ts}* females, raised to adulthood at the permissive temperature, do not display male courtship behavior. However, if they are shifted to the restrictive temperature for several days, some male courtship behavior can be observed. This suggests that the functional state of the adult CNS is under permanent control of the sex-determining genes and is not irreversibly fixed at some earlier stage.

These examples raise the question what the term "sex-determination" actually means. In a classical sense, the sex of a cell appears never to be determined, but rather is reversibly locked in one of two possible "conditions". Changing a genetic signal downstream from the X:A ratio at any time in development affects the state of activity at the subordinate control genes, with the domino effect finally reaching the differentiation genes. The experiments with the temperature-sensitive mutation *tra-2^{ts}* show that the change in gene activity can go in both directions, from female to male and from male to female.

1.6. Molecular analysis of the regulatory genes

The molecular analysis of some of the regulatory sex-determining genes that have been cloned so far (*Sxl*, *tra* and *dsx*) reveals a complex pattern of transcription (Maine et al., 1985a; 1985b; Belote et al., 1985b; Butler et al., 1986; Baker et al., 1987; McKeown et al., 1987). Whereas the temporal expression of these genes is largely consistent with genetic data, the appearance of non sex-specific and male-specific transcripts for *Sxl* and *tra* was not expected.

For the *dsx* locus which is under the control of *tra*, *tra-2* and *ix* (see Fig.1.), a non sex-specific transcript is present till the end of the third larval period; then sex-specific transcripts begin to appear. The female- and male-specific mature mRNAs consist of three exons common to both sexes and a 3'exon that is different in females and males (Baker et al., 1987). Since XX animals that are mutant for *tra* or *tra-2* are transformed into males, it appears that *tra* and *tra-2* are necessary to set the *dsx* gene in the female-specific expression state.

The gene *transformer* on the third chromosome at 73A9-10 was cloned by microdissection, deficiency mapping and chromosome walking techniques (Butler et al., 1986), and it could be shown that the function of *tra* is harboured within a 2kb DNA fragment which can restore the female phenotype when introduced into XX; *tra/tra* homozygous flies (Butler et al., 1986; McKeown et al., 1987). We were interested in the structure and precise expression pattern of the *tra* gene that is regulated by *Sxl* and is involved in the regulation of the sex-specific expression of the *dsx* locus.

2. METHODS

2.1. DNA preparation methods

2.1.1. Small scale preparation of plasmid DNA

Bacterial cells from 5ml cultures were pelleted in a Sorvall RT6000 at full speed for 15min. The pellets were resuspended in 200 μ l 50mM Tris-HCl pH 7.5, 10mM EDTA and 1mg/ml lysozyme (freshly prepared). The content was incubated at room temperature for 10min. SDS was added to 0.5%, the content swirled and incubated on ice for 30s. Potassium acetate was added to 0.4M, the content swirled and incubated on ice for 30min. The cell debris were pelleted in an eppendorf centrifuge at 4°C for 15min. To the supernatant pancreatic RNase (heat treated) was added to 25 μ g/ml and the content incubated at room temperature for 10min, followed by a phenol extraction, a chloroform extraction and precipitation with isopropanol (hydrous solution/isopropanol 1.25/1).

2.1.2. Large scale preparation of plasmid DNA.

Bacterial cells from a 250ml culture were pelleted at 8000rpm (GSA-rotor), 4°C for 15min. The pellet was resuspended in 2ml of 50mM Tris-HCl pH 7.5. Then 500 μ l of a freshly prepared solution of lysozyme (10mg/ml) was added, the content swirled and incubated 30min on ice. 400 μ l of 0.5M EDTA pH 8 was added. The content was mixed and incubated on ice for 5min. 80 μ l of RNase (10mg/ml) was added, the content mixed and incubated on ice for 5min. 60 μ l of 10% Triton X-100 was added, the content mixed and incubated on ice for 30min. The genomic DNA was pelleted at 17000rpm (SS34-rotor), 0°C for 80min. The supernatant was extracted twice with phenol and twice with chloroform and precipitated twice with 12.5 volumes of ethanol out of 0.3M sodium acetate.

2.1.3. Small scale lambda DNA preparation

Using a purified clone, confluent plates were prepared and overlaid with 5ml of 10mM Tris-HCl pH 7.5 and 2mM $MgCl_2$. The plates were stored at 4°C for 16h. The overlay was diluted with 5ml of 10mM Tris-HCl and applied to a 2ml DEAE-cellulose column which has been washed with 4ml of 10mM Tris-HCl pH 7.5. The DEAE-cellulose had previously been washed sequentially with 2ml of 100mM HCl, 2ml of 100mM Tris-HCl pH 8.0 and 2ml 10mM Tris-HCl pH 8.0, with 5ml of 10mM Tris-HCl pH 8.0 and 60mM sodium acetate. Then 800µl of elution buffer (10mM Tris-HCl pH 8.0, 50mM magnesium acetate) was added, the flowthrough discarded and 1.2ml of elution buffer added. This flowthrough was collected and lambda DNA was prepared by adding 120µl of TES buffer (100mM Tris-HCl pH 8.0, 100mM EDTA, 1% SDS) and 20µg of proteinase K and incubated at 37°C for 1h. The DNA was phenol/chloroform/isoamylalcohol (25:25:1) extracted, chloroform extracted and precipitated with ethanol out of 0.3M ammonium acetate.

2.1.4. Large scale lambda DNA preparation

4×10^9 pfu (plaque forming units) were adsorbed to 400µl of bacteria (JM101 OD₅₅₀ = 0.6) in SM buffer and incubated at 37°C for 20min (2% gelatine, 1M Tris pH 7.5, 0.2% $MgSO_4$, 0.58% NaCl). 200 ml of NZYM medium (1% NZ amine which is type-A hydrolysate of casein from Humko Sheffield Chemical Division of Kraft, Inc.; 0.5% NaCl; 0.5% yeast extract; 0.2% $MgSO_4$; adjusted to pH 7.5 with NaOH) was added and 50ml cultures were shaken vigorously for 16h. 5ml chloroform was added to each culture and shaken for 15min. The cell debris were centrifuged at 5000rpm for 15min and to the supernatant excluding any remaining chloroform, 200µg of pancreatic RNase and 200µg of DNase I was added and

incubated on ice for 1h. 12g of NaCl and 20g of polyethylene glycol 6000 were added, incubated on ice for 1h and centrifuged at 5000 rpm. The supernatant was discarded and the bottle kept inverted for 15min. The phage pellet was dissolved in 2X500 μ l of SM and centrifuged for 5min in an eppendorf centrifuge. 10 μ l of 0.5M EDTA and 10 μ l of 20% SDS were added to the supernatant, incubated at 65°C for 15min and extracted carefully twice with phenol/chloroform/isoamylalkohol, once with chloroform and precipitated with 1 volume of isopropanol out of 0.3M sodium acetate.

2.2. Subcloning of DNA fragments in pUC or pBR322

DNA from λ or plasmid preparations was digested with the appropriate restriction enzyme as recommended by the suppliers. Digested fragments were separated on (low temperature) agarose gels, isolated, ligated with either pUC or M13 vectors and transfected into E.Coli.

2.2.1. Low melting temperature agarose gels

To purify labeled or unlabeled DNA fragments, 0.8-1.5% low melting temperature agarose gels containing 0.5 μ g/ml ethidium bromide, submerged in TAE buffer (0.04M Tris acetate, pH 7.5, 0.002M EDTA) were used. The gels were cast and run in the cold room. The DNA (0.2 to 1 μ g) to be purified was loaded in a loading buffer containing 0.04% bromophenolblue, 0.04% xylene cyanol, 0.2mM EDTA and 5% glycerol. The gels were run at 8 V/cm. Gel slices containing the appropriate fragments were cut under UV-light, weighted, NaCl (1 M) was added to 0.5M and the slices were incubated at 65°C for 10 min. The DNA was extracted once

with phenol which had been preheated at 65°C, once with chloroform and then ethanol precipitated, the pellet washed in cold 70% ethanol and dissolved in TE.

2.2.2. Ligation of DNA fragments to plasmid vectors

For subcloning, 20 ng of plasmid vector, digested with the appropriate restriction enzyme, were incubated with a 2 to 3 times molar excess of DNA fragment in presence of 10 mM rATP, 10 mM Tris-HCl, pH 7.5 and 10 mM MgCl₂. For a reaction volume of 20 µl, 1 to 2 units of ligase were added and the sample was incubated at room temperature for 5 hours. After the ligation reaction the samples were kept at 4°C.

2.2.3. High-efficiency transformation of E.coli

For a transformation efficiency of $1 \cdot 4 \times 10^8$ resistant colonies/µg pBR322 the following procedure was used: One colony was picked from a fresh plate and dispersed into 1ml L-Broth (LB) medium by vortexing and incubated with agitation at 37°C for 4 to 20h. This culture was used to inoculate a prerinsed flask of LB medium (100ml in a 1000ml flask, 50ml in a 500ml flask). The culture was incubated at 37°C, 275rpm (Kuehner shaker), until cell density was about 4×10^7 (absorbance at 550nm = 0.2 for JM101 in about 1-2 h; Yanisch-Perron et al., 1985). The cells were collected into 50ml Corning tubes, placed on ice for 15min and pelleted at 2500rpm in a Sorvall RT6000 for 15min at 4°C. The cells were resuspended in 8.5ml TFB by passing the suspension several times carefully through a pasteur pipet, placed on ice for 10min and pelleted again at 2500rpm for 10min at 4°C. TFB (transformationbuffer) is 10 mM K-MES (pH 6.2), 100 mM RbCl, 45 mM MnCl₂, 10 mM CaCl₂, 3 mM HAcCoCl₃. The pH was adjusted to 6.15 with 0.1 M

KOH and the buffer sterile-filtered through a prerinsed Millipore filter (0.22 μm) and stored at 4°C. 1 M MES is adjusted to pH 6.3 using KOH, sterile filtered, and stored at -20°C. All salts were added as solids. The pellet was resuspended in 2ml TFB. Freshly thawed DMSO was added to 3.5%, swirled and left on ice for 5min. DTT was added to 75mM, swirled and left on ice for 10min. Another equal portion of DMSO was added, swirled and the cells left on ice for 5min. Samples of 200 μl were placed into chilled Falcon (2059) tubes containing DNA in less than 10 μl and the mixture was incubated on ice for 30min. The mixture was heat-pulsed without agitation at 42°C for exactly 90s and chilled on ice for 2min. Then 800 μl of LB medium (room temp.) was added and the tubes were incubated at 37°C, 225rpm for 1h (Hanahan, 1983). 100 μl were pipetted onto a LB plate containing appropriate antibiotic and spread with an L-shaped glass rod. The plates were incubated at 37°C for about 20h or until the colonies had a diameter of 1mm. Colony density was about 300-1000 colonies/plate. If necessary the cells were pelleted and resuspended in a smaller volume before spreading to adjust colony density.

2.2.4. Calcium-chloride transformation method

For a transformation efficiency of about 10^6 resistant colonies/ μg pBR322 the following less sophisticated method was used: From a cell culture in LB medium with a cell density of about 4×10^7 cells/ml, 20ml were collected into a 50ml Corning tube and incubated on ice for 10min. The cells were pelleted at 3000rpm in a Sorvall RT6000 for 10min at 4°C. The cells were resuspended in 6.8ml 50mM CaCl_2 and incubated on ice for 1h. Then the cells were pelleted at 2500rpm, 4°C for 5min and resuspended in 1.6ml of 20% glycerol in 50mM CaCl_2 , incubated on ice for 10min and placed in -70°C. For transformation the cells were thawed on

ice or used directly before placing in -70°C . $100\mu\text{l}$ of cells were added to falcon tubes containing $100\mu\text{l}$ of annealed DNA dissolved in TCM (10mM Tris-HCl pH 7.5, 10mM CaCl_2 , 10mM MgCl_2). The content was incubated on ice for 30min, incubated at 42°C for exactly 60s and incubated at room temperature for 10min. 1ml of LB was added and the content incubated at 37°C for 60min without agitation. The cells were spread on LB plates as described above.

2.3. Screening of Lambda libraries

10^3 plaque forming units (pfu) of the lambda library were adsorbed to $100\mu\text{l}$ of bacteria (JM101 $\text{OD}_{550}=0.6$) in $500\mu\text{l}$ of SM buffer (100mM NaCl, 10mM MgSO_4 , 50mM Tris-HCl pH 7.5 and 0.01% gelatin) for 20min at 37°C . 3ml of NZYM medium top agar (7g/l bacto agar) cooled to 55°C was added and distributed on a 85mm plate which was dehydrated at 37°C for 1h. The plates were kept at room temperature for 30min and incubated at 37°C for 16-20h. The density was approximately 1000 plaques/plate. Nitrocellulose filters (82mm) were prepared. The filters were hybridized to DNA probes; the filters were prehybridized in 50% deionized formamide, $5\times\text{SSC}$, $5\times\text{Denhardt's}$ solution ($50\times$ Denhardt's is 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA in distilled H_2O), 50mM NaPO_4 , 1% glycine pH 6.5 and $200\mu\text{g}/\mu\text{l}$ denatured sonicated salmon sperm DNA in a covered plastic container at 42°C for 4h. The denatured probe was added and the filters were hybridized for 16h at 42°C . The filters were washed twice at room temperature (rt) with $2\times\text{SSC}$ for 10min, twice at rt with $1\times\text{SSC}$ for 10min, once at rt with $0.5\times\text{SSC}$ for 10min and once at 60°C with $0.5\times\text{SSC}$ for 20min. The filters were exposed wet (wrapped in Saran) to an X-ray film with an intensifier screen at -80°C . For rehybridization, the filters were washed in water at 100°C for 15min and then prehybridized and hybridized as described above.

If it was not possible to pick discrete crosshybridizing plaques, a region was picked with the large end of a pasteur pipette and the top agar transferred to 100 μ l of SM buffer. Serial dilutions of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} were prepared. 10 μ l of each dilution was adsorbed to 100 μ l of bacteria (JM101 OD₅₅₀=0.6) and plated as described above. Filters were prepared and hybridized. Crosshybridizing plaques could be recovered cleanly from lower dilutions and were transferred to 500 μ l of SM buffer and kept at room temperature for 30min. 100 μ l of bacteria (JM101 OD₅₅₀=0.6) were added and incubated at 37°C for 20min. 2ml of NZYM medium was added and shaken vigorously overnight at 37°C. Chloroform was added, shaken for 15min and centrifuged in RT6000. The titer of the supernatant was determined and the amplification repeated until the titer was 10^9 - 10^{10} .

2.4. Radioactive labeling of DNA fragments

2.4.1. Labeling of DNA by "nick-translation"

Labeling of DNA was done by nick-translation with a kit obtained by NEN in a volume of 25 μ l containing 100mM dATP, 100mM dGTP, 100mM dTTP, Tris-HCl pH 7.8, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 50 μ Ci (α -³²P)dCTP, 2mg/ml DNA, 520 units DNA polymerase I/ml and 10 μ g DNase I/ml at 15°C for exactly 2 h. EDTA was added to 20mM and the DNA separated from unincorporated nucleotides by gel filtration on Sephadex G-50 (pasteur pipet) which was equilibrated in TNE (10mM Tris, 100mM NaCl, 1mM EDTA), 150 μ l fractions were collected. The peak-fractions (usually 4 or 5) were pooled and tRNA was added to 110 μ g/ml. The DNA was precipitated with ethanol, the pellet washed with cold 70% ethanol and dissolved in TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA).

2.4.2. Radioactive labeling of single-stranded DNA probes

This procedure should generate single-stranded DNA of fairly high specific activity corresponding to an insert cloned into M13. Also a probe made this way will have a sequence identical to that read directly from a sequencing gel, and it will hybridize to an RNA having the complementary sequence. 100 ng DNA, approximately 10% of a standard M13 single-strand miniprep as used for sequencing (see 2.9.3.), were diluted to 25 μ l. 5 μ l of a solution of universal primer (0.5 pmol/ μ l) were added and 5 μ l 10X Klenow buffer (100 mM Tris, pH 7.5, 600 mM NaCl, 66 mM $MgCl_2$). After annealing at 65°C, 6.5 μ l "nick mix" was added (5 mCi/ml $\alpha^{32}P$ -dATP and $\alpha^{32}P$ -dCTP -800 Ci/mmol- plus 20 μ M dGTP and dTTP; 20 mM DTT) and 1 μ l Klenow (5 U/ μ l) and incubated at room temperature (rt) for 20 min, and another 10 min at 37° C. 15 μ l chase mix (250 μ M each dATP, dCTP, dGTP and dTTP) incubated at rt for 20 min.

To create a single-stranded fragment of defined length, digestion with the appropriate restriction enzyme is necessary. The probe is then diluted with loading buffer (10 mM EDTA, 1 % SDS, Xylene Cyanol, bromphenol blue in DMSO) and boiled for 3 min, then loaded on TAE (40 mM Tris-acetate, 2 mM EDTA) low gel temperature agarose gels and the radioactive probe is isolated as described 2.2.1.

2.5. Southern Blots

Up to 1 μ g of recombinant DNA which had been restricted with appropriate restriction enzymes was fractionated on 0.8-1.5% agarose gels without ethidium-bromide submerged in TAE buffer. The DNA was transferred by the capillary blot procedure: Gene Screen Plus filter was cut to the size of the gel, wetted in distilled

water, layed onto a 10xSSC solution (1.5M NaCl, 0.15M sodium citrate) and allowed to soak for 10-15min. A support (1cm thick) was layed into a tray, filled with 10xSSC to less than 1cm. A glass plate of at least the size of the gel was placed on the support and covered with filter paper (Whatman 3MM) which hung over into the solution. The gel was placed onto the filter paper. On the gel the wet Gene Screen Plus filter was placed so that the side B was in contact with the gel. On top 3-6 pieces of wet filter paper, 6-10 pieces of dry filter paper (Whatman 3MM) (cut to the same size of the gel) and 10-15cm of absorbant paper towels (cut in the same size as the gel) were placed. The transfer was allowed to continue for 1-2h (small DNA molecules), the filter rinsed in 2xSSC and dried. Hybridization to ^{32}P - oligonucleotides or ^{32}P -DNA was done under the same conditions as described in (2.6).

To rehybridize the filter with DNA, it was incubated in 200ml of 0.4N NaOH at 42°C for 30min and in 200ml of 0.1 X SSC, 0.1% SDS and 0.2M Tris-HCl pH 7.5 at 42°C for 30min. If too much probe was left, the washes were repeated. The filter was then prehybridized and rehybridized as described above.

2.6. RNA extraction

Guanidinium isothiocyanate stock (5 M) was prepared by mixing 250g of Fluka purum grade guanidium thiocyanate with 21ml 1M Tris-HCl and 8.5ml 10mM EDTA. Deionized water was added to approximately 400ml and the solution was stirred for 4h and filtered. 21ml of 2-mercaptoethanol was added, its pH was adjusted to 7 with a small amount of 5N NaOH and the volume of the solution was adjusted to 420ml with deionized water. Cesium chloride stock (5.7 M) was prepared by mixing 192g of Fluka purum grade cesium chloride with 40ml 0.5M

EDTA. Deionized water was added to 200ml and its pH was adjusted to 7.4 with acetic acid. The solution was filtered through millipore filter and the refractive index was adjusted to 1.400 (25°C) with deionized water.

Crystalline phenol was distilled at 180°C, 8-hydroxyquinoline was then added to a final concentration of 0.1%. The phenol was extracted 3 times with an equal volume of 1M Tris-HCl pH 8, then extracted 3 times with 0.1M Tris-HCl pH 8 and 0.2% 2-mercaptoethanol.

2 g of frozen tissue were dropped into 20ml of guanidinium isothiocyanate stock solution in a 50ml corning tube and homogenized immediately for 60s at full speed with a 12mm diameter Polytron homogenizer. 0.9g of sodium lauryl sarcosinate and 10ml of cesium chloride stock (5.7 M) were added. The homogenate (30ml) was then layered onto 5ml of a cesium chloride stock (5.7 M) cushion in a polyallomer tube (25.4x85 mm) and the tube was centrifuged in a Sorvall AH627 rotor at 24000rpm for 20h at 20°C (Chirgwin, 1979). After centrifugation, the supernatant was removed carefully with a 5ml disposable pipet, the tube inverted and all but the bottom (1.5cm) was sheared off. The RNA in the pellet was then dissolved in an appropriate volume of TE (10mM Tris-HCl pH 7.6, 1mM EDTA) by passing it through a pipet until the RNA pellet was completely dissolved.

Poly(A)⁺ RNA was selected on oligo(dT)-cellulose (BRL or CR type 3) (Aviv and Leder, 1972). 0.1-0.2g of the cellulose was suspended in 0.1N NaOH and packaged in a sterile glass column, washed with 15ml 0.1N NaOH, 30ml binding buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 0.5M NaCl, 0.5% sodium lauryl sarcosinate). The RNA (<1mg/ml) was incubated at 65°C for 5min, chilled on ice and an equal amount of 2x binding buffer was added. It was applied twice to the column. The nonabsorbed material was eluted by washing with 25ml of binding buffer, the material retained by the column was eluted with 3ml elution buffer (10mM

Tris-HCL pH 7.5, 1mM EDTA and 0.05% SLS) and ethanol precipitated out of 0.3M sodium acetate, washed in cold 70% ethanol and the pellet dissolved in TE (3-7 μ g/ μ l).

2.7. Northern Blots

RNA was fractionated on 1% agarose gels containing 17% formaldehyde and submerged in MOPS buffer (40mM morpholinopropanesulfonic acid pH 7, 10mM sodium acetate, 1mM EDTA). The buffer was recirculated and stirred. The gels were 4mm thick and the combs had a surface of 4x1mm. The gels were run at 3 V/cm for 5-6 h. The RNA (usually 3-10 μ g) was mixed with a solution containing 0.8xMOPS buffer, 42% formamide, 8% formaldehyde, and H₂O in a volume of 25 μ l. To denature the RNA, the content was heated to 60°C for 5min and chilled on ice. Formamide was deionized (Mixed bed resin AG 501-X8(D)) for at least 1h. 5 μ l of loading buffer (50% glycerol, 1mM EDTA, 0.04% bromophenolblue) was added. At the end of the run, the gel was stained with ethidium bromide (1 μ g/ml) for 20min, photographed and incubated in 50mM NaOH for 30min. The transfer to Gene Screen Plus filter was done as described in (2.5.). The filter was rinsed in 2xSSC, air-dried and baked at 80°C for 2h. If ³²P-DNA was used as probe, the filters were prehybridized in 0.5M Phosphate buffer pH 7.2 (Na₂HPO₄ adjusted to pH 7.2 with phosphoric acid), 1% BSA, 1mM EDTA and 7% SDS at 65°C for 30min and hybridized in the same solution at 65°C for 16-40h. If ³²P-oligonucleotides were used as probes, the filters were prehybridized in 0.5M Phosphate buffer, 6% SDS, 0.3M NaCl, 1mM EDTA and 1% BSA at 10°C below melting temperature (T_M) for 20h and hybridized in the same solution at the same temperature for 40h. To the hybridization solution tRNA was added to 20 μ g/ml.

The probes were heated to 100°C for 5 min and chilled on dry ice before they were added. The wash solutions were preheated to 65°C, and the washes were performed at room temperature. The filters were washed sequentially with different solutions: When cDNA was used as probe, washes were performed once in 40mM Phosphate buffer, 5% SDS, 1mM EDTA, 0.5% BSA for 5min, 4 to 8 times in 40mM Phosphate buffer, 1% SDS and 1mM EDTA for 3min, 3 times in 100mM Phosphate buffer pH 7.2 for 3min and once in 100mM Phosphate buffer for 20min at 65°C. When oligonucleotides were used as probes, the washes were done in the same way as described above, but 5 M NaCl was added to 0.7M and the temperature was 10°C below T_M . If necessary, additional washes at higher temperatures were performed for higher stringency. The filters were exposed to X-ray films using intensifier screens at -70°C.

If the filters were rehybridized, they were never dried completely. The membranes were washed 4 to 5 times in 150ml of 0.01% SDS and 0.01xSSC at 95°C for 2min. The washes were repeated if too much probe was left. The filters were prehybridized and then rehybridized as described above.

2.8. Reversed Northern analysis

DNA preparations of λ phages were digested with restriction enzymes and fractionated on agarose gels (as described in 2.5.) and blotted onto nylon filters.

2.8.1. c-DNA synthesis

Annealing of the oligo dT to p(A)+RNA was carried out in a volume of 40 μ l containing 50mM sodium phosphate pH 7.5, 5mM EDTA, 100-300 μ g/ml RNA and 1.5 μ g/ml of oligonucleotide for 3 min at 90°C, KCl was added to 100mM, the mix

cooled down slowly for 5min to approximately 60°C and chilled on ice for 5min. Synthesis of first strand cDNA was carried out in a reaction volume of 100µl containing 70mM KCl, 100mM Tris-HCl pH 8.3 (42°C), 10mM MgCl₂, 0.4mM DTT, 1mM dGTP, 1mM dATP, 1mM dTTP, 1mM dCTP and 300 units reverse transcriptase/ml for 2 h at 42°C. The reaction was stopped by adding EDTA to 24mM. The products were extracted with phenol/chloroform (1:1) and chloroform. An equal volume of 4M NH₄ acetate and two volumes of ethanol were added. The products were precipitated for 10min on dry ice, warmed up to room temperature by gently mixing and centrifuged for 10min in an Eppendorf centrifuge at room temperature (Okayama and Berg, 1982). The pellet was washed in cold 70% ethanol, dried in vacuum and dissolved in TE.

2.8.2. Hybridization of Southern Blots with cDNA

c-DNA probes were denatured for 3 min in boiling waterbath and added to pre-hybridized filters, incubated at 65°C. Hybridization was performed at 65° C for 24 hours. Washing conditions were the same as described in 2.5.

2.9. DNA sequencing

2.9.1. Creation of Bal 31 deletions for random sequencing

4 to 8 µg plasmid DNA were digested at the restriction site which was chosen to generate Bal 31 degraded random ends (Legerski et al., 1978). Aliquots of DNA were tested for total restriction digest on analytical gels. The digested DNA preparation was extracted with phenol and chloroform and precipitated with ethanol. For Bal 31 digestion, the DNA was dissolved in 20 µl distilled H₂O and 20 µl 2x Bal 31 buffer (1.2 M NaCl, 24 mM CaCl₂, 40 mM Tris pH 8, 2 mM EDTA) were added. Bal 31 reaction was carried out at 30°C and with 2 units Bal 31 (Boehringer).

Aliquots of 10 or 20 μ l were taken after 3, 6, 9 min. of incubation. The reaction was stopped by immediately extracting the degraded DNA samples with phenol, chloroform and additional ethanol precipitation. After digestion with a second restriction enzyme, the heterogeneous fragments were separated on low temperature agarose gels and gel extracted (as in 2.2.1.).

2.9.2. Cloning of DNA fragments in M13

All cloning steps for sequencing were performed after the protocol of Pharmacia (Sanger et al., 1980; Yanish-Perron et al., 1985). M13 vectors mp8, mp9, mp18 and mp19 were used. For Bal 31 created fragments either Sma I or Hinc II digested vectors were produced. All restriction enzymes were supplied by either Boehringer or Pharmacia.

2.9.3. Preparation of single-stranded M13 DNA

Single M13 plaques were picked with sterile toothpicks and grown in 1.7 ml 2x TY (0.5% NaCl, 1% Tryptone, 0.5% yeast) medium with 170 μ l of a fresh over night culture of JM 101 or JM 109 cells (Yanisch-Perron et al., 1985). After 6 hours of vigorous shaking, the culture was transferred to Eppendorf tubes and centrifuged for 5 min. The supernatant was transferred to new tubes and 200 μ l of PEG (20 % PEG, 2.5 M NaCl) added and the samples were incubated at RT for 20 min, and centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was pipetted away carefully and the PEG pellet was resuspended in 100 μ l TE, extracted with phenol and precipitated out of 0.3 M NaAc and 2.5 volumes of ethanol. The DNA pellet was dried and resuspended in 50 μ l TE. For sequencing, 8 μ l of this standard preparation were used.

2.9.4. Sequencing reactions

Sequencing reactions were performed after the procedure described by Pharmacia (for sequencing with $^{35}\text{SdATP}$) and all reagents were supplied by Pharmacia. Sequencing reactions were carried out in microtiter dishes and 20 clones were sequenced at a time.

2.9.5. Sequencing gels

6% acrylamide, 7M urea in 1x TBE (90mM TrisHCl, pH7.5; 90mM boric acid, 20mM EDTA) gels were poured. Sequencing gels were of two different sizes, 30 X 40 and 20 x 40 cm. The broad gel system was used for sequencing 20 clones at a time. Gels were 0.3 mm thick and slots were 2 mm broad. Probes were loaded with drawn capillaries. Gels were run at 35 to 40 mA and 1300 to 1500 V. Gels were fixed in a 10% acetic acid, 10% methanol solution for 15 min, dried and exposed on X-ray films.

2.10. S1 Mapping

Probes used for S1 mapping were created as described in 2.9.2. and single stranded DNA was prepared as described in 2.9.3. The amount of RNA was either 10 μg total or 2 to 5 μg of p(A)⁺ RNA per sample.

2.10.1. RNA/DNA Hybridization

RNA and 0.4 pmol DNA (0.1 μg single-stranded M13 template) were resuspended in 50 % formamid, 1 M NaCl, 10 mM Tris-HCl pH 7.4 in a final volume of 10 to 50 μl . Samples were incubated over night or at least 5 hours at 50°C. As a

control probe without RNA and different RNA species, not containing probe complementary sequences were also incubated (e.g. E.Coli RNA).

2.10.2. S1 digestion

After hybridization 9 volumes of S1 buffer (0.35 M NaCl, 1 mM ZnCl₂, 0.03 M NaAc, pH 4.6, 5% glycerol) were added and approximately 50 units S1 (Pharmacia) and samples were incubated at 37°C for 15 min. and afterwards precipitated with ethanol.

2.10.3. Separation on sequencing gels.

S1 digested samples were carefully resuspended in formamide loading buffer. Separation was carried out on 7 M urea, 6% acrylamide gels (sequencing gels) which were dried and exposed with intensifying screens at -70°C over night.

2.11. "In situ" Hybridization

All steps were performed after the protocol of E.Hafen (1983).

2.11.1. Preparation of Tissue Sections

Sections of frozen tissue were cut with a SLEE cryo-microtome. Adult flies, larvae or embryos were embedded in O.C.T. and frozen either on dry ice or in liquid nitrogen. Sections were absorbed on gelatinized microscope slides until an area of 18x18 mm was covered. Microscope slides were then placed for 2 min. on a heat block of 50°C and air dried for at least 20 min. at rt.

2.11.2. Fixation of sections

Slides were placed into a slide rack and immersed in a solution containing 4% paraformaldehyde in PBS. Slides were incubated in this solution for 20 min. The slides were then incubated for 3 min. in 3x PBS and 2 times in 1X PBS for 5 min. To dehydrate, the slides were incubated in a graded series of ethanol: 30%, 2min, 60 %, 2 min, 80 %, 5 min, 95 %, 2 min, 100 %, 2 min. Afterwards the slides were air dried.

2.11.3. Preparation of Nick-Translated Hybridization Probes

Hybridization probes of optimal size are of 50 to 100 nucleotides single-stranded. The probe size depends on DNase I concentration and the most reproducible results were obtained with DNase I from Worthington Biochemicals (DPFF grade). To obtain the proper size, we performed three nicktranslation reactions with three different concentrations of DNase I. DNA used for hybridization was either purified plasmid or gel purified fragment.

250 to 500 ng of DNA were incubated with 25 μ Ci of each α^{32} P-dATP, α^{32} -dCTP, α^{32} P-dGTP and α^{32} P-dTTP, lyophilized. (If not all nucleotides are added, the final concentration of cold nucleotides should be 25 μ M). The reaction was carried out with 1x nick-translation buffer (0.5M Tris, pH7.2; 0.1M MgSO_4 ; 1mM DDT), 10 units of E. coli DNA polymerase I and three different concentrations of DNase I (1ng, 2ng, 4ng). Tubes were incubated at RT for 45 min. The reaction was stopped by adding 25 μ l of 50 mM EDTA. The percentage of incorporation was measured by TCA precipitation of 1/10 of the reaction.

2.11.4 Hybridization of Tissue Sections

Sections were prehybridized in 2 X SSC at 70°C, once for 5 min and again for 30 min, then rinsed in distilled H₂O. Sections were dehydrated as in 2.11.2. and air dried. Dry sections were layed out on a black surface, and 5 µl of hybridization probe in hybridization mix (1M NaCl, 5x Denhardt's, 0.1% SDS, and 10% dextran sulfate) were applied to each slide, covered with a coverslip and sealed with rubber cement. The microscope slides were then stored in a wet chamber and incubated for 24 hours at 35°C.

After hybridization, the rubber cement was peeled off and slides were submerged in a beaker containing wash solution at 35°C (50 % formamide, 0.6 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA). Wash solutions were changed three times in the first hour and another three times within the following 24 hours. Slides were again dehydrated by incubation in 70% ethanol (2 times 5 min.), 95% ethanol (2 times, 5 min) and air dried at RT.

2.11.5. Autoradiography of Tissue Sections

Autoradiographic emulsion (Kodak NTB-2, CAT 165 4433) was divided into aliquots of 5 ml. For 20 to 50 slides, one aliquot was melted at 45°C in the dark-room (Kodak safe light filter No 2/ Cat 152 1525,15 V light bulb). Emulsion was filled in a dipping chamber and sections are carefully individually dipped into the chamber and placed on a cooled metal plate to harden the emulsion immediately. After drying for 2 hours at rt, sections were exposed at 4°C for 3 to 30 days.

2.11.6. Developing of slides

The slide box was warmed up to RT . Slides were placed into developing solution equilibrated at 15 °C for 2 min (Kodak D-19 developer Cat 146 4598). Slides were transferred to a 2% acetic acid solution precooled to 15°C and incubated for 30 sec. Slides were fixed in precooled Kodak fixer for 1 min. Afterwards slides were rinsed for 15 min in three changes of distilled water precooled to 15°C. Then the slides were incubated for 30 sec. in precooled Giemsa (5% in 10mM NaPO₄, pH 6.8) solution for 30 sec. Slides were rinsed by dipping 3 times in distilled water, air dried and mounted.

3. RESULTS

3.1. Experiments to define the *tra* gene

The *tra* gene was cloned by microdissection and chromosome walking. The borders of the gene were defined by mapping deficiency breakpoints and spontaneous lesions (Butler et al., 1986).

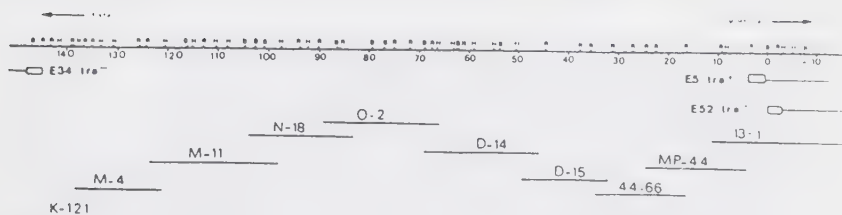


Fig.2. Map of the chromosomal walk.

The map is calibrated in kb, and restriction enzyme sites are symbolized by: R: *EcoRI*, B: *BamHI*, H: *HindIII*. Representative phage and cosmid clones are shown below the restriction map. The breakpoints of three deficiencies are given (boxes) with the solid lines indicating DNA present. E5 is a *tra*⁺ line and E34 and E52 are *tra*⁻.

At a time when only the breakpoints of the deficiencies E34 and E52, two *tra*⁻ mutations, limited the locus to 140 kb, we tried to identify the *tra* gene by means of differential transcription in females and males, because the genetic data indicated

that the function of the *tra* gene was not necessary in males. We carried out dot blot analysis with DNA of the λ phages covering the 140kb chromosomal walk and with female and male RNA. Hybridization was shown with DNA of phages K-121, M-4, MP-44 and 13-1 for females and males. We then performed the more sensitive method of reversed Northern hybridization with retrotranscribed poly(A)⁺RNA from females and males. Recombinant λ phages carrying overlapping DNA sequences covering the region of 140 kb, were digested with Eco RI or Hind III, and the resulting DNA fragments were blotted onto nylon filters and hybridized to cDNA either from females or from males.

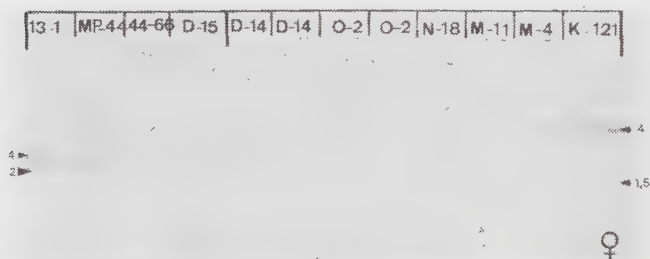


Fig.3. Reversed Northern Analysis.

DNA of λ phages 13-1, MP-44, 44-66, D-15, D-14, O-2, N-18, M-11, M-4 and K-121, was digested with EcoRI or HindIII, fractionated by electrophoresis on agarose gels and transferred to nylon filters using the Southern (1975) procedure. DNA of λ phages 13-1 to D-15 was fractionated on a different gel than DNA of λ phages D-14 to K-121. The filter was hybridized to ³²P-cDNA from female poly(A)⁺RNA and autoradiographed. Hybridization signals that are larger than 4kb derive from partial digestion. Hybridization with male cDNA gave the same results.

Again two recombinant phages at the distal end of the walk, K-121 and M-4, showed hybridization for a 1.5kb and a 4kb Eco RI fragment both in females and

males. At the proximal end of the walk, transcription was detected in the recombinant phages 13-1 and MP-44. A 4kb and a 2kb Eco RI fragment were labeled with both cDNA species. No fragments were exclusively labeled with female-specific c-DNA. Between map point +120 and +25 (Fig.2) no transcripts were detected of either sex.

Later on, mapping the breakpoint of deficiency E5 which is *tra*⁺ allowed Butler et al. (1986) to delimit the *tra* locus to 8kb within λ phage 13-1 (Fig.2). The DNA aberrations of two spontaneous mutations *tra*¹ and *tra*^{Z4}, additionally could be mapped within a 2kb BamHI-EcoRI fragment of λ phage 13-1. The final proof that this region contains the functional *tra* gene was obtained by germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). A 6kb BamHI fragment and a 3.8kb EcoRI fragment, both from the proximal part of the walk (λ phage 13-1), were inserted into P-transformation vectors (Steller and Pirrotta, 1985). The transformed lines were tested to see whether the inserted DNA could rescue the *tra* phenotype (Butler et al., 1986). Lines transformed with the 3.8 kb transposon showed complete rescue of the *tra* phenotype, while lines with the 6 kb transposon exhibited only partial rescue. The transposon containing the 6kb DNA fragment showed different degrees of rescue, depending on the site of integration.

3.2. Transcription pattern of the *tra* region

We further analysed the RNA species transcribed from the *tra* region and surroundings, as defined by the 3.8kb fragment (see chapter 3.1) on Northern blots. RNA species of five size classes (4 kb, 1.9 kb, 1.6kb, 1.3 kb and 0.9 kb) were detected in adult females (Fig.4).

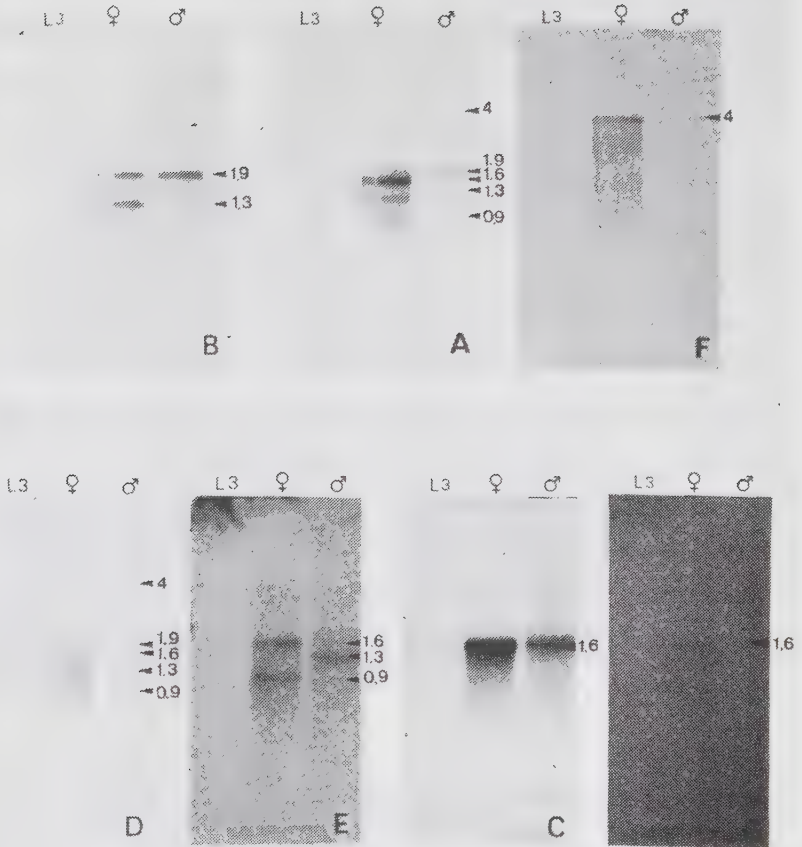


Fig.4. Northern blot analysis.

Poly(A)⁺RNA (5-10 µg per lane) from adult females (♀) or males (♂) or unsexed third instar larvae (L3) was electrophoresed on agarose gels, transferred to nylon filters and hybridized to nick-translated DNA fragments or labeled single-stranded DNA probes, representing the intervals shown in Fig.5. Panel E represents a filter hybridized to a single-stranded probe derived from fragment D (left to right), Panel G represents hybridization with a single-stranded probe from fragment A (right to left); transcripts of 1.9kb and 0.9kb in panel G probably result from previous hybridization of the same filter with fragment D.

The 4-kb RNA is transcribed from right to left in the map shown in Fig.5 and clearly initiates outside the region that contains a functional *tra* gene as defined by the 3.8kb DNA fragment (chapter 3.1). It is therefore unlikely to be involved in *tra* function. Two female-specific RNAs of 0.9kb and 1.6kb are transcribed within fragment D from right to left. This was shown by hybridization with a single-stranded probe made from fragment D. A male-specific RNA is transcribed from the same strand within fragment D (Fig.4E). Another transcript of 1.6kb derives from fragment C and is transcribed from left to right. It is more abundant in females than in males. This was shown by hybridization with a single-stranded probe made from fragment A (Fig.4G), and hybridization with double-stranded probes C and A.

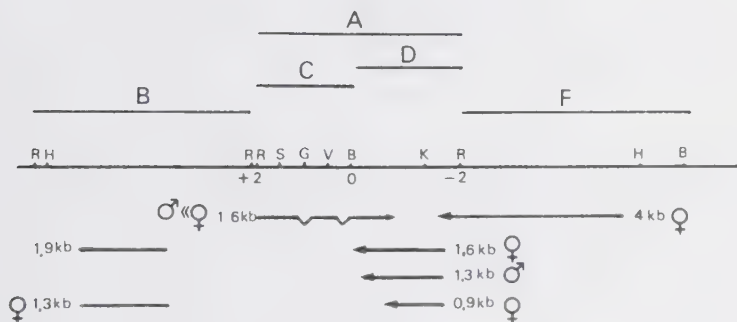


Fig.5. Transcript map of the *tra* region.

The map is calibrated in kb. Restriction enzymes are symbolized by: R: Eco RI, H: Hind III, S: Sph I, G: Bgl II, K: Kpn I, V: Eco RV, B: Bam HI.

RNAs of 1.9 and 1.3kb are seen with both probes D and B, but not with probe C. It is possible that these transcripts contained introns spanning the entire 0 to +2 interval and transcribe only small exons within fragment D. RNAs of these two sizes are seen in both males and females using probe A (3.8kb Eco RI fragment) and probe D (the 0 to -2 interval), but the 1.3-kb species seen in the male must be a different transcript from that seen in the female, since it is not detected with probe B. This is confirmed by hybridization with the single-stranded probe E that reveals the male, but not the female 1.3-kb species. A single-stranded probe (of the opposite strand) spanning the Bam HI site, detects only the 1.6kb RNA (Fig.4G), which is more abundant in females than in males and in larvae and is transcribed from left to right (Fig.5.).

The most likely interpretation for the detection of the RNA species of 1.9 and 1.3kb with fragment D is sequence homology between fragment B and fragment D. With single-stranded probes from fragment D these transcripts do not light up.

In summary, these results suggest that seven different RNA species are encoded by the region surrounding the *tra* locus (Fig.5). Three of these, of 4kb, 1.6kb and 0.9kb, are predominantly or exclusively female, and one of 1.3kb is male-specific. The 1.6kb and 0.9kb female-specific transcripts and the 1.3kb male-specific transcript derive from the *tra* gene. These findings were supported by transformation studies that showed that fragment D (0 to -2) contained all functional sequences of the *tra* gene (Butler et al., 1986; McKeown et al., 1987) and by DNA aberrations of spontaneous mutations that map within fragment D

3.3. cDNA clones from the 3.8kb tra region

To elucidate the primary structure of the female-specific transcripts, we screened two different λ gt10 cDNA libraries derived from RNA of adult females (Poole et al., 1985) to find the corresponding cDNA clones. We isolated six different clones, when screening with the entire 3.8kb EcoRI fragment. Three of them crosshybridized with fragment C and fragment D (Fig.6). We further analyzed these and an additional pupal cDNA clone (provided by V.Pirrotta), isolated from a pupal-specific cDNA library, not sexually specified.

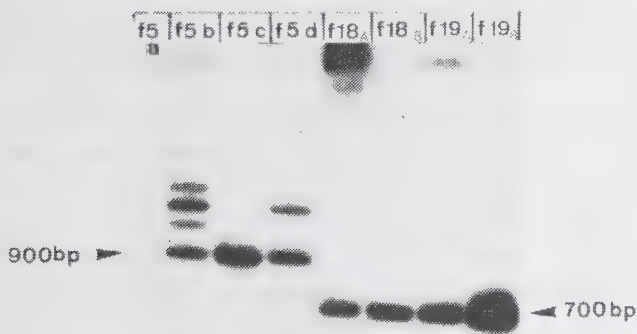


Fig.6. λ gt10 cDNA clones.

cDNA clones, EcoRI digested, gel fractionated, are hybridized with genomic fragment D. Clones f5a,b,c,d, are different preparations of the same clone, as are f18A and B, and f19 A and B. Fragments of cDNA clones f5 (a,b,c,d) that are larger than 900bp result from partial digests. cDNA clone f5a was lost during the preparation.

Restriction analysis indicated that the largest part of each clone was homologous to genomic region C (Fig.5). None of the clones crosshybridized with fragment D only. The RNA detected by the pupal cDNA clone or by the female cDNA clones f18 and f19 corresponds to the 1.6kb RNA species (Fig.8b) that derives from fragment C (Fig.4C). This 1.6kb RNA species is a few times more abundant in females than in males. With the female cDNA clone f5, a 4kb and a 2kb RNA species were detected. Sequence analysis of three female-specific and one pupal cDNA clone was carried out. Two of the female clones proved to be nearly identical (f18 and f19), except at their 3'end. The third female clone (f5) consists of multiple EcoRI fragments (Fig.6), suggesting that its sequence extends outside the 3.8kb EcoRI fragment.

The pupal clone (pc) is colinear with the female clones f18 and f19, but different at its 3' and its 5'end. The approximate extension at the 5'end was estimated by restriction analysis. The pupal clone and female clone f5 did not contain proper 3'ends, probably as a cloning artefact during the construction of the cDNA library. Therefore, only the 3' end of f18 (Fig.7B) indicates the real 3'end of the corresponding transcript. Clone f5 additionally consists of sequences lying outside the 3.8kb genomic fragment (sequencing data not shown). The most abundant transcripts that are detected with clone f5 are 4kb and 2kb.

Comparison of the genomic sequence of the 3.8kb EcoRI fragment and the cDNA sequences showed that f18, f19 and pc share at least three exons of a transcription unit. The direction of transcription is from left to right in the map of Fig.7B and was indicated by consensus splice donor and acceptor sequences and by the 3'end of clone f18. The resulting transcript extends from fragment C over the BamHI site into fragment D. It overlaps the sequences of the putative *tra* transcription unit (see Fig.9 and chapter 3.4.) with at least 50 bp and terminates at position 1521 (see Fig.7 and Fig.9) on the genomic sequence of the *tra* region.

CCGAGTGCCTGTACAAGCTGCGGAGCAITCCCAGTAATCGGAAGCGAGCGCATCCGCCG... 60
 TTTTGAGCGTTCCGCGCGGGAGTGGGAAATCGAAAAATCCACAGCAGCAAGCCATCGTC... 120
 CGCTGCAAAATAGGGATAAATAGGATATAGTAACGTAGTTCAAGCACACAATCGGGGGGC... 180
 CAGCATGGAGCGGCGCGTGAAGCTGAAGACGATCAAGTCCACATAACGTGCAAGATCCCC... 240

120 bp

GAGATCTGCGGCGGCTACTTCATCGATGCCACCTCGAAGACCTGCCCCACCTGCGACAAC... 300
 ATCATCCATCAGTCCACCCCTGCAATACATCAGCTTCGATCGCACCAATGCAGGACATG... 360

75 bp

TGTACAACTGGTGCCGAACTGCAAGAAGATGAATCGCGCCGAGAGCGAGACTTCTACA... 420
 AGACAGGAACATGCCGTGTCCCAAGGATATCACGCAGAACGCAGATGATAATGAGAAGTG... 480
 CGGACGCCACGCCGAGCCGAGCCGACTCCATCGCCTGGACGAGCAGGTGAACGTGTGCC... 540
 GGAGTGCAATTAGCAATAACTTCAAGAACCTGCAGAGGCGATTTCGTGCGCGTCAGCTCGCA... 600
 GCGACGATAACGCATCTCAAGAAGCTGGTGGCCCAAGAAGATCCTCAACGGCACCGAAAA... 660

118 bp

GTACAGAGAGATCGACATACTGTGCAACGAGGAGCTGCTCGGCAAGGATCACACGCTGAA... 720
 GTTCGTCTACGTGACCTGGCGCTTCCGGGATCGGGGATCCGCTTCGGTGACAGTTTCGCT... 780
 CCGCGGGTCGAGCTGGCCTAACAAGTGTTTTAGTATAATGCCACAACAAATGATCTATTT... 840
 TTATAATTTATTGACAAATGCGTAAATATCGTAATTAGGAGAGTCGCTAGTGTAGTCCCC... 900
 TCGAAACCATGAGTGTATGTGTAATGTGCATGTATAAAGTCGTAATATATGGTTATCC... 960
 CTCCTAGAAATTAAGTAACCTCCACTTCCTAACTCGTGTGACCCCTTTTTTTCGCCCTTCT... 1020
 TTGCTGCATTTGCTCATGATTTAATCAACGTTCTCCGTTTGCAATATTTATGTCTCCA... 1080
 TTTTAGTTTGAAATATATATGTATTTATTATTTTAAATGTACAAAAACACGGAATGTAAT... 1140
AAATTTGTAATTATGTCGAA.....

Fig.7A. Nucleotide sequence of cDNA clones pc, f18, f19 and f5 (transcribed from left to right in Fig.5).

Position 755 corresponds to Bam HI site (at position 1922 in the nucleotide sequence of the tra region). Underlined sequence indicates overlapping regions with the putative "tra" ORF B2 (see Fig.9).

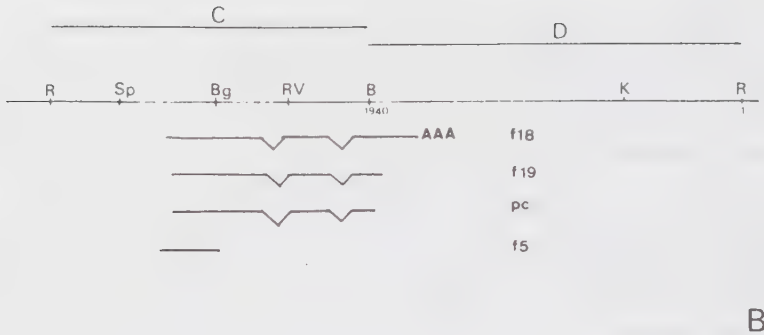


Fig.7B. Transcription map of the vicinity of *tra*. Sequences of the cDNA clones are aligned with the genomic sequence. (*pc*: pupal, *f18*, *f19* and *f5*: female).

3.4. Nucleotide sequence of the *tra* region

The genomic sequence of the *tra* region, as defined by the 3.8kb *EcoRI* fragment, was determined by the Sanger dideoxynucleotide chain termination technique (Sanger et al., 1977). Restriction analysis of fragment C and D showed convenient restriction sites in fragment C that could be used for direct cloning of restriction fragments into M13. For fragment D, subclones were created by unidirectional deletions by *Bal31* nuclease (Fig.10). The regions of overlapping DNA were established by sequence comparison with DNA sequencing programs DBAUTO and DBUTIL (Staden, 1980). Part of the composite sequence is shown in Fig.9. The sequence from position 1 to 1521 was deduced solely from genomic clones, while the region between position 1521 to 3600 was sequenced both from cDNA clones and genomic clones (genomic region from position 1922 to 3600 was sequenced by U.Ernst, unpublished).

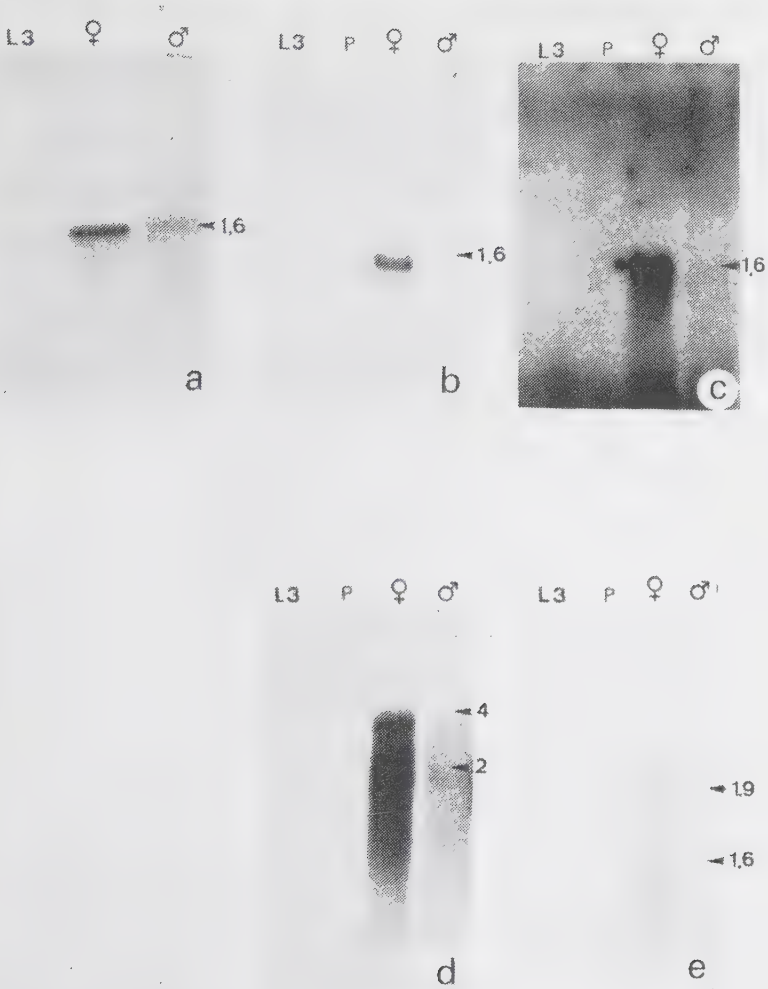


Fig.8. Northern blots probed with cDNA clones of the 3.8kb *tra* region. Poly(A)+RNA (5-10 μ g per lane) from adult females (♀) or males (♂) or unsexed third instar larvae (L3) or unsexed pupae (P) was electrophoresed on agarose gels, transferred to nylon filters and hybridized to nick-translated cDNA of clone *f18* (b), *f5* (d) and *pc* (a and c) (male RNA is underrepresented in b and c). Panel e represents the same filter as d hybridized with the genomic 3.8 *Eco*RI fragment as used in Fig.4A.

The sequence was analysed for potential protein coding regions and possible polyadenylation and mRNA processing sites, using the program ANALYSEQ (Staden, 1984a, 1984b). We found several open reading frames (ORF), that appeared likely to be coding sequences. Within positions 2000 and 3600 (or 0 to +2; in Fig.5.), three ORFs were identical with exons that were already identified by sequence comparison of genomic and cDNA sequences (chapter 3.3). Between positions 1 and 2000, five ORFs with possible protein coding sequences were identified. The first ORF (C), from position 1 to position 313, ends with a stop codon, followed by a polyadenylation signal at position 427 and 438 (Proudfoot and Brownlee, 1976). This ORF is probably part of the 4kb transcript detected on Northern blots with genomic fragments F, A and D (Fig.4, chapter 3.2.).

The longest ORF, A2 (525 bp; positions 797-1321), has an amber termination codon at its 3'end, but no polyadenylation (AATAAA) signal. But there are several less conserved splice donor sequences (Mount, 1982) and it is therefore possible that ORF A2 is spliced to ORF A3 (166bp; position 1361-1527) and terminates with an amber stop codon, followed by a polyadenylation signal at position 1572. The 5'end of ORF A2 has a good splice acceptor sequence. The only possible 5'exon with a methionin translation start codon is indicated by ORF A1 (30bp; 624-654) that is preceded by a TATA box at position 501. The composite transcript of ORF A1, A2 and A3 would have a total length of 971bp, consisting of about 88bp untranslated leader and 75bp trailer sequence.

A second large, potentially protein coding ORF, B2 (426 bp; positions 1140-1566), starts with a consensus splice acceptor sequence at its 5'end and has a termination codon at its 3'end. A possible polyadenylation signal could be used at position 1830. There is also high protein coding probability for a small exon with a methionine translation start codon, ORF B1 (64bp; positions 945-1009), with a

GGAGTGGGCIGTTCGCCAC¹GGCCACTTGTGTCGGTAACCAACAGCITTCIGTTCAGGAG... 60
 CTCATCGAGGTACATGCAATGTGCCGATTTGGTGGCAATCAGTCCAGTGGTGGTAC... 120
 GATCCCCGGATGGGCGGCACGGGGAGATCGATGTGGCGGTGCTCCGGTCAACGAGGA... 180
 GGAFTTGTCTGAAAGGGAGCGGCTCATTTGGTGGTCTTCAATGGAAAACGAGCGCATCAGTGT... 240
 CCGGATAGTGTAAATTTAAATTTGGTGTATTTGGTGGTCTATCTGGTCTTCTTA... 300
 TGTGTGTAGTTAGTTAAACAAAATTTGTATGTGTACTGTGAATGAAACCGTGTGGCAGAG... 360
 TATTGTAAGAAACGCATAACCCATAAGTTAAATTAATCTCGTTGTTCCTAAAACCTGAAT... 420
 TAGCTTAAATAAAACTAAATAAGTATATGAGTTAATTTTAAATTTGATATGTTGCTCT... 480
 CGAATAATCAAAAAGTAACGTATATGTAGCGATAGTTCTGTCGAAAAGTGAGTTGGCGGG... 540
 ACATTGCAAGTCGAGCAAGCGAGTACGGTCACACTGAGGAAAGTGCATCATTTAATTTTC... 600
 CAGCATTTTCCGATCGAAAATGGAT^{A1}TCGCACAGCAGTGGAAACCCAGCATCGAGGTACCAT... 660
 ATATAGTATAACCGATCTGATCAGATCTGATCCAGTGGCATCTTTTGGTGTGTTT... 720
 CTAGTGTCTATTTGTGTGAAATGTGAAATGGATGAGAAATGAACGCTGGACGACCAGACAG... 780
 CAGAAAGAGAAGCTAGGACAATAGGACT^{A2}TCTCAACTGCCGATTACGTGGATTCGGTCTCCG... 840
 M...R...Q...I...L...C...
 ACGATGGCCCAACACTATCGCTTAGATGCATTGACTAACCAGCACACTCTTTTACATAG... 900
 B1
 M...G...D...Q...A...
 ATTTCCGTGGCTCAAGGTCTCGATCCCGGGCAGAAAGAGAATACATGGCGCATCAAGCGA... 960
 B1
 R...G...T...A...E...R...R...S...T...R...F...R...I...L...Q...T...R...
 GAGGGACAGCAGAAGAAGGAGCACAAGATTCGGTACTTTGCAGAGCAGGTTGAGAA... 1020
 GGATCGGTGTGAGAAGATTGGCGCAAGAGCGGACCAATCCACAGACGCACTCGCTCCAG... 1080
 ATCCAGATCAGAGTCGTCCATCAGAGAGAGCAGGACACAGAAGGCATCGCAGCGTCTAGG... 1140
 B2
 F...P...Q...S...Q...P...I...V...F...A...V...N...F...N...A...V...N...G...A...F...
 AGCCGCAATCGCAGCCTGAAGTCGAGGCACTGAACGAAACGCCGTCAACGGAGCGGAAG... 1200
 V...A...A...V...N...F...D...A...V...N...G...A...R...I...G...I...I...L...R...Q...
 TCCGAGCAGTGAACGAAGAGCGGCTCAACGGAGCCGCATCGGTATTAATCTCTCCGCAAA... 1260
 R...S...S...F...F...M...C...K...C...H...H...R...I...S...T...L...V...V...I...C...
 GATCATCACTACTATGTGCAAGTGGCACCACAGGATCTTACGTGAGTAGTATTTGTGA... 1320
 I...E...L...N...K...S...N...I...N...R...I...C...R...G...C...I...V...C...S...
 TTGAAACAAAATAAATCTAATTAATCGATTTTGCAGGGGATGTTGGTGGTATGTCAGGA... 1380
 A2
 K...V...L...L...D...T...K...G...Y...H...V...I...R...R...F...H...R...P...P...I...D...
 AAGTTTGGGATACCAAGGCTACACAGTCTCCGGCGTTCACCGGGCCCTTACAGATA... 1440
 A3
 I...A...S...D...R...R...S...I...I...I...R...D...I...A...I...I...I...R...G...V...
 CCGCCAGCGACCGCGGCTCATTTGGAGTTCGGGATTTGGCTACAGAAACCGGGGGCTTC... 1500

P H I L I P P I N I F A C P
 CCATATGAACTACTCCATTCGACATAATACAAATTAATACATTCGGTGTGTTG 1560
 V. H. .
 TACATTAATAAATAAATAACATAATATTCAAACTAAAAATGGAGAACAATAAAATATG 1620
 CAAACGGAGAACGTTGATTAAATCAAGGCAAAATGCAGCAAGAAGGCGGCAAAAAAAGG 1680
 GTACACGAGTTAGGAAGTGGAAAGTTACATAATTCTAGGAGGGATAACCATATATTTACG 1740
 ACTTTATACATGCACATTTACACATACACTCATGGTTTCGAGGGGACTAACACTAGCGAC 1800
 TCTCCFAATTACGATATTTACGATTTGTCAATAAAATTATAAAAAATAGATCATTGTGTG 1860
 GCAATTATACATAAACACATTGTTAGGCCAGCTCGACCCGCGGAGCGAAACCTGCAGAAAGCG 1920
 GCGGATCCCGATCCCGGAAGCG

Fig.9. Nucleotide sequence of the *tra* region (2kb D fragment). Nucleotide sequence from position 1 (*EcoRI*, corresponding to -2 on the map in Fig.5.) to 1922 (*BamHI*, corresponding to 0 in Fig.5.). TATA boxes, AATAAA sequences and splice junctions are underlined. ORFs are symbolized by circles at their 5' start positions and rectangles at their 3' ends. Sequences deduced from cDNA clones are underlined.

splice donor consensus sequence at its 3' end. The transcript composed of ORF B1 and B2 would result in a length of 970 bp, including a 250 bp leader and 306 bp trailer sequence. Instead of ORF B1, the methionin start codon at position 844 (B1') could be used, spliced at position 862 which would result in a continuous ORF together with B2.

Another possibility would be the splicing of ORF A1 to B1 and subsequently to B2, or directly to B2. For the 0.9kb and 1.6kb female-specific and the 1.3kb male-specific *tra* transcripts we found only two possible promoter consensus sequences in fragment D downstream from TATA boxes at positions 501 and 659 and two polyadenylation signals at positions 1572 and 1830.

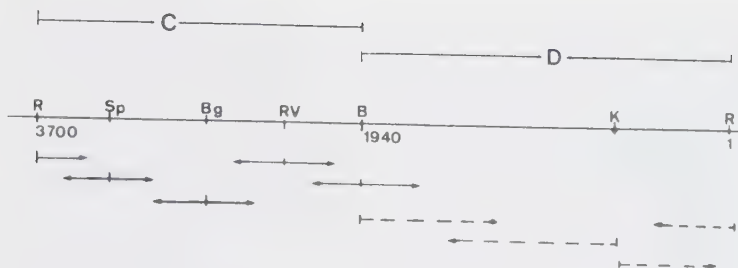


Fig.10. Sequencing strategy for the 3.8kb EcoRI fragment.

Restriction sites, used for M13 cloning and subsequent sequencing of genomic and cDNA clones are indicated and symbolized: R: EcoRI; B: BamHI; Sp: SphI; Bg: BglII; RV: EcoRV; K: KpnI. Interrupted lines represent subclones of heterogeneous length, which were created by the Bal31 nuclease degradation technique.

3.5. S1 Mapping of the putative tra transcript

We tested the genomic region between positions 652 and 1565 for differential transcription in females and males by S1 mapping. Four different single-stranded probes were constructed (Fig.11) one probe, A, containing the DNA sequence between positions 1 and 661 (A), probe B containing sequences from positions 661 to 1720, and two clones containing the opposite orientation. S1 mapping analysis with clone A showed a predominant protected fragment of 440bp. This RNA was present in early embryos (24 hours), second and third instar larvae and in adult females and males (Fig.11). In males, however, it was two to three times less abundant. The size of this fragment could result from protection of an RNA that initiates outside the 3.8kb Eco RI fragment and probably terminates after the

polyadenylation signal at position 428. ORF C described in chapter 3.4. could be part of this transcript.

Single-stranded probe B shows protection of a fragment of approximately 670 bp (Fig.11) when total RNA was used. The protected band of 670 bp is present in larval RNA, in adult females and in *traZ⁴* pseudomales. It is not or only at a very low abundance present in males. The size of this protected fragment is consistent with the predicted ORF B2. An RNA transcribed from that sequence could be polyadenylated at position 1860 and would result in a protected fragment of 720bp. ORF A2, spliced to A3, would result in two protected fragments of 513 bp and 241 bp, ORF A2 alone, if the polyadenylation signal at position 1571 were used, would result in a protected fragment of 805 bp. We therefore conclude that ORF B2 represents the *tra* transcript.

But our results do not allow us to identify the 5'exon(s) from which transcription actually starts. The fact that a 1.6 and a 0.9kb RNA is transcribed in females makes a precise S1 mapping more difficult. There are less prominent protected bands visible in different stages of larvae and adult females when probed with single-stranded probe B. However, their size (467bp and 360bp) is difficult to explain at the moment. Recent S1 mapping experiments with smaller fragments covering the region between position 656 and 1350, also did not identify a 5'exon but nevertheless confirmed ORF B2.

With both probes A and B, RNA from adult homozygous *traZ⁴* pseudomales was also tested. The spontaneous mutation *traZ⁴* is known to result from a 200 bp DNA insertion within fragment D (genomic region 0 to -2) (Butler et al., 1986). The size of the protected band with probe A is smaller for this RNA species, indicating that the DNA insertion of this mutation lies near the 3'end of ORF C (positions 1 to 310). When tested with single-stranded probe B, RNA of this mutant shows the

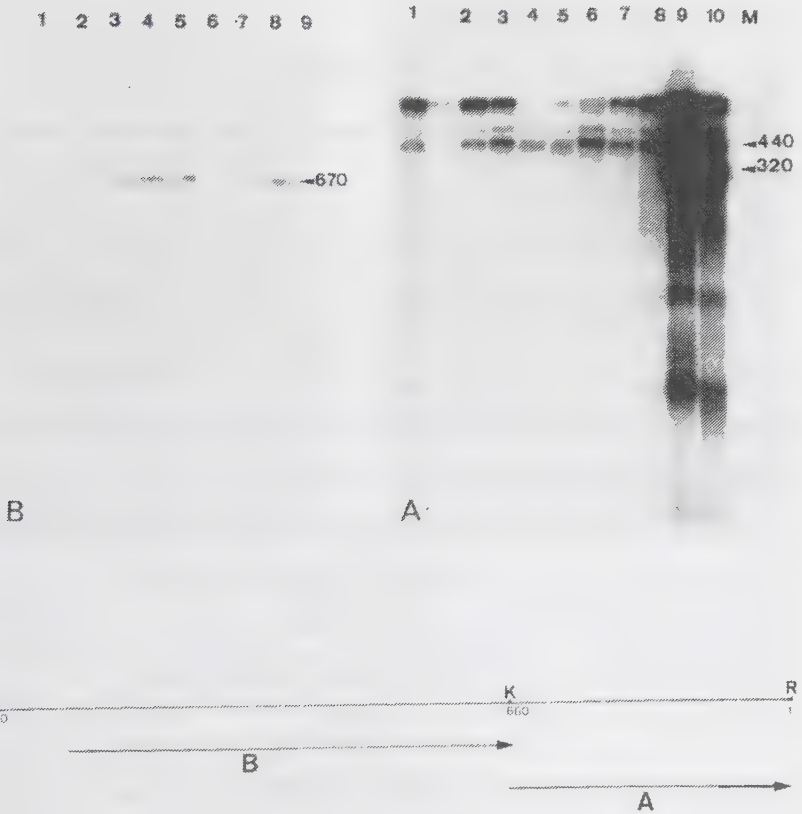


Fig. 11. S1 mapping of transcripts in fragment D (0 to -2 in Fig. 5). Panel A and B represent S1 mapping experiments with single-stranded probes, produced as indicated in the drawing below. 10 to 20 μ g total RNA for panel B and 3 to 10 μ g poly(A)⁺RNA in panel A (except 9 and 10, where total RNA was used), were loaded to each lane. RNA probes (A): embryos, 24 hours (1), 48 hours (2), third larval stage (3), pupae (4), different male RNA preparations (5, 7), different female RNA preparations (6, 8), *tra*^{Z4} homozygous pseudomales (9), *tra*^{Z4} hemizygous pseudomales (10). RNA probes (B): 1, 2, 3, 4, like in A, female (5, 8), male (6, 9) *tra*^{Z4} pseudomales (7), size marker (M) (Retardation in (2) caused by different salt concentration in the loading buffer).

same protected band as RNA of normal females, but less abundant. As a control for the S1 mapping of single-stranded probes A and B, we tested the opposite strand, and no protection was seen.

3.6. Proposed amino acid sequence deduced from the nucleotide sequence

The longest ORFs, A2 and B2 (Fig.9) within fragment D, code for a protein of 175 amino acids and 142 amino acids, respectively. The 142 amino acids of ORF B2, which we tentatively identified by S1 mapping to be transcribed as female-specific RNA, contain 7 cystein residues and 26 positively charged residues. Even when a potential 5'exon which harbours the translation start is added, the resulting protein will not become larger than 160 amino acids.

A computer search of the Protein Information Resource (PIR) data base, Dayhoff Protein data base and EMBL protein data base for ORF B2 was performed in an attempt to identify sequence homologies between the putative *tra* gene product and other proteins. No obvious homologies were found so far.

3.7. Developmental expression of the *tra* transcripts

As a result of genetic experiments it was postulated that the *tra* functions are needed throughout development (Baker and Ridge, 1980; Epper and Nöthiger, 1982; Wieschaus and Nöthiger, 1982). This conclusion was based on the observation that homozygous *tra*⁻ clones, induced by mitotic recombination result in a mutant phenotype even in late larval stages. Molecular data now confirm this hypothesis. Both Northern blot analysis and S1 mapping show that female-specific

transcripts from the *tra* locus (the 2kb D fragment, Fig.5) are present in all larval stages, as well as in pupae and adult females (Fig.11).

3.8. Search for tissue- and sex-specific expression of *tra*

RNA from the *tra* locus is present in higher abundance in adult females than in larval stages. Since the RNA was extracted from larvae that were not divided by sex the lower rate of RNA in larvae could result from the 50% males which do not make the same transcript. Another explanation would be a change in tissue specificity during development.

With "in situ" hybridizations we wanted to answer several questions. First we wanted to see whether the 0.9kb and 1.6kb female-specific transcript of the *tra* region (fragment D, position 659 to 1922, covering the putative *tra* ORF B2; see Fig.9) is specifically expressed in females. We also considered that hybridization to male tissue could appear, corresponding to the 1.3kb male-specific RNA that was detected with a single-stranded probe of fragment D. We were further interested to know whether *tra* was expressed in all cells or restricted to specific tissues. From genetic experiments we tend to favor the former possibility, but the possibility remains that in larvae the functions of *tra* are only expressed by cells of the imaginal discs. We hybridized sections of female and male larvae and adult females and males with a *tra*- DNA probe that spans the region from position 661 to 1780. However, with this *tra* probe, we could not detect any specific hybridization neither in adult flies nor in larvae. The expression of the 1.6kb and 0.9kb RNA from the *tra* locus (fragment D) is very low and might be difficult to be detected by "in situ" hybridization, unless it were restricted to specific cells. Our results therefore are negative evidence for the proposed ubiquitous expression of the *tra* gene.

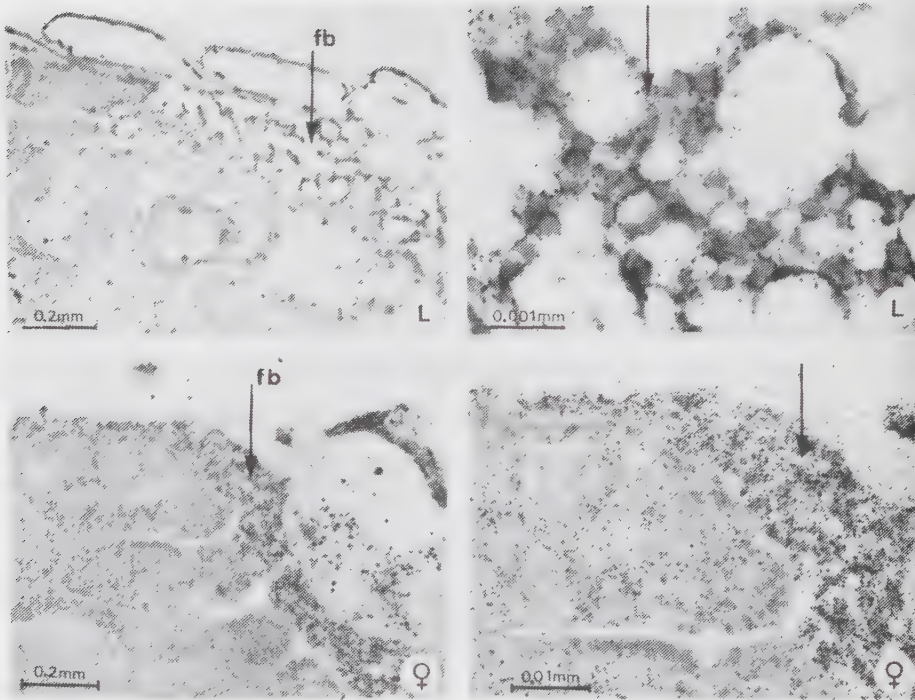


Fig. 12. "In situ" hybridizations.

Female larvae (L) and adult females (♀) were hybridized with ^3H nick-translated DNA of cDNA clone pc (Fig. 7B).

The left side represents a 40 fold, the right side a 160 fold magnification. Arrows indicate labeled tissue (fb: fatbody). All sections shown are from female individuals; males show the same hybridization pattern.

Second, we wanted to get more information about the 1.6kb transcript that derives from fragment C (Fig. 5) and overlaps the putative *tra* ORF. It is a few times more

abundant in females than in males and is represented by the cDNA clones in Fig.7B. As a probe we used the pupal clone, pc that does not contain the 3'end of the 1.6kb transcript and ends at position 1914 near the Bam HI site (Fig.9). With this probe it could be shown that this transcript is expressed in the cells of the larval and adult fat body in both sexes (Fig.12). This result clearly eliminates the 1.6kb transcript that derives from fragment C (Fig.5) as a candidate for the expression of *tra*- like functions, or products regulating the processing of the *tra* products.

4. DISCUSSION

The ultimate goal of this work is to elucidate how *tra* specifies sex-determination at the molecular level. This effort obviously requires more information about the structure, expression and distribution of the *tra* product(s).

4.1. Transcriptional activity at chromosomal region 73A

When the region of the 140 kb chromosomal walk, at the cytological location 73A, was tested for differential transcription, it was striking that from +25 to +115 (see Fig.2 and Fig.3) no transcripts were found. In contrast, at least seven different transcripts map within a region of 12 kb. These transcription units are not only extremely crowded, but even overlapping (see Chapt. 3.3.). It is a known feature of the *Drosophila* genome organization that there is less transcriptional activity where few bands are visible on salivary gland chromosomes, and more in a region of densely packed bands (Bossy et al., 1984). The *tra* gene also maps near a strong band on salivary gland chromosomes at position 73A9-10.

4.2. Localization of the functional *tra* gene

The best evidence for location and size of the *tra* gene was obtained from germline transformation. It was possible to restore the wildtype functions in homozygous *tra/tra* mutants with transposons that contained the 3.8kb Eco RI fragment (A in Fig.5). In lines transformed with a transposon containing fragment

C plus B, the *tra* phenotype could not be rescued; but a transposon spanning the region of fragment F plus D could largely restore the wildtype functions, depending on the site of integration (Butler et al., 1986). It was obvious that the functional gene therefore lies within fragment D, and it was reported by McKeown et al. (1987) that 2kb of DNA, identical to our fragment D, were in some cases capable of rescuing *tra*. The different degrees of rescue could mean that not all necessary regulatory sequences of the gene are contained within fragment D, and it is only activated when incidentally integrated downstream of promoter sequences that, however, must allow the properly regulated expression of *tra*. Alternatively, it is possible that all sequences necessary for the correct expression of the *tra* gene are within fragment D, but that at some chromosomal sites the transposon is subject to position effects.

The analysis of the transcripts that are homologous to fragment D showed that the adjacent transcription units on both sides of the *tra* gene terminate within fragment D. It is therefore more likely that all the regulatory sequences of *tra* are very close to the transcribed region of *tra* and not beyond the adjacent genes.

4.3. Transcripts from the 2kb fragment that contains the *tra* locus

Probing Northern blots of poly (A)+RNA with single-stranded DNA from fragment D, we could show that three different transcripts derive from this stretch of DNA (Fig.4), namely a 1.6kb and a 0.9kb female-specific transcript, and a 1.3kb male-specific transcript.

To the right (5')side of *tra*, a 4kb transcript appears that initiates within fragment F and is much more abundant in females. It is transcribed from the same strand as *tra*.

To the left (3')side of the *tra* gene, a transcript of 1.6kb, initiating within fragment C and having the same size as the larger female-specific *tra* RNA, can be mapped. But this transcript is in an antisense orientation to the *tra* transcripts. This is supported by probing Northern blots with a single-stranded probe spanning fragment A (Fig.4G) and by sequencing data from cDNA clone f18 that represents this 1.6kb RNA (Fig.7A). Using double-stranded DNA probes (fragment C,D) we could not distinguish the transcript initiating in fragment C from the 1.6kb female-specific *tra* transcript.

The hybridization of fragment A (Fig.4) to the 1.9kb non sex-specific RNA and the 1.3kb female-specific RNA that were detected with probe B (Fig.4) is probably the result of crosshybridization to transcripts that derive from fragment B. These transcripts are at least 20 times more abundant than the *tra* transcripts (exposure time of filter E in Fig.4 was 2 hours while the other filters were exposed for 1 to 3 days)

4.4. Does the 3'flanking transcript influence the expression of *tra*?

From our sequence analysis of the genomic region and of cDNA clones representing the transcript on the left (3')side of *tra* we know that the 3'end of cDNA clone f18 and the 3'end of the putative *tra* ORF overlap at least 50bp. Could this have a regulatory influence on the expression of *tra*? If both genes are transcriptionally active in the same cell, RNA hybrids might be formed that could alter RNA processing events.

We could show by "in situ" hybridization that the gene that maps to the 3'side of *tra* is transcribed in the fat body of both sexes in larvae and adult flies (Fig.12).

Thus, any regulatory influence of the 1.6kb transcript that derives from fragment C, on the stability or the processing of the *tra* transcript would be restricted to the fatbody. Since mutations in *tra* have global effects on all aspects of sex, the exclusive expression in the fatbody is not compatible with the functions of the *tra* products. Unfortunately, the expression of the *tra* gene is too low to be detected by "in situ" hybridization. But relying on genetic data suggesting that the products of *tra* are required in all cells of a female, during larval development and throughout the adult period, we argue that there is probably no regulation or influence between the two neighbouring genes.

4.5. Proposed structure of the *tra* gene

The structure of the *tra* gene could only be deduced from genomic DNA sequences. Although we screened three different cDNA libraries, one pupal and two made from adult females, we could not identify a clone that correlates to the transcripts of the *tra* gene. One reason might have been the more abundant transcript at the 3'side of *tra*. Screening with the 3.8kb Eco RI fragment as hybridization probe resulted probably in weaker signals for a *tra* cDNA clone then for a cDNA clone from the neighbouring 1.6kb transcript.

The genomic region within fragment D shows the 3'end of the 4kb transcript to the right (5'side) of *tra* as the end of an open reading frame (ORF) at position 310. The transcribed region and the regulatory sequences of *tra* therefore lie within approximately 1600 bp. Two TATA boxes can be identified at a distance of 100 and 300 bp from the potential polyadenylation signal of the 4kb transcript (Fig.9).

ORF B2 described in chapter 3.4. is transcribed in larval and pupal stages and in adult females and probably at a very low level in males. Several small exons with methionine translation initiation codons can be deduced from the DNA sequence between position 400 and 1000 (Fig.9). We could not determine from which sequences transcription actually starts. The S1 mapping results, however, allow us to determine the 3'end of the *tra* gene at the polyadenylation signal at position 1850. The genetically defined *tra* function is probably represented by the 0.9kb transcript that is female-specific. It is possible that transcription of this small transcript starts after the TATA box at position 660. The larger transcript in females and the 1.3kb male-specific transcript could result from the use of a different transcription initiation site or from differential processing.

If we consider the role of *Sxl* in female individuals, and if we argue that *Sxl* acts directly or indirectly on *tra* by activating transcription (Maine et al., 1985a; 1985b), the *Sxl* -dependent *tra* transcript should be present in females only. We then must conclude that the female and male transcripts are transcribed from different promoters, the inducible one in females and the *Sxl* -independent one in males. Alternatively, the products of the superior control gene *Sxl* could be involved in the sex-specific processing of a *tra* RNA precursor common to both sexes. To verify the first possibility, it will be necessary to precisely map the *tra* promoter(s) and to study its (their) specificity in vivo.

4.6. Function of the *tra* product

The products of the *tra* gene are only necessary in female individuals. XY flies that are homozygous for *tra* are viable and fertile males. The female-specific 0.9kb transcript, consistent with genetic data, is probably responsible for the

female-specific action of *tra*. The 1.6kb female transcript might either play a role by interacting with the female-specific 0.9kb transcript or it has other cryptic functions that escape our detection.

The protein deduced from ORF B2 consists of 142 amino acids (Fig.9) and is highly polar. We could not find significant homologies to other known proteins.

Genetic evidence suggests that the function of the *tra* product is to regulate the expression of the subordinate regulatory gene *dsx*. This gene expresses a product F in females and a different product M in males. In the absence of *tra* or *tra-2*, XX individuals fail to produce the female determining F factor and instead display the male phenotype as a result of the production of M, the male determining factor of the *dsx* locus (Baker and Ridge, 1980; Nöthiger and Leuthold et al., 1987).

Recently, the *dsx* gene, a large locus of some 35kb, has been cloned, and the transcription pattern of the *dsx* locus in females and males was analyzed (Belote et al., 1985; Baker et al., 1987). Beginning with the pupal stage, different RNA species can be detected in females and males. The female and male RNAs consist of four exons, three are common to both sexes, but the 3'exons are sex-specific. The male-specific 3'exon lies 3000 bp downstream of the female-specific 3'exon.

For a functional *dsx^f* product, the activity of *tra*, *tra-2* and *ix* are necessary. If we assume that the two sex-specific RNAs of the *dsx* locus correspond to the genetic functions F and M, we could deduce that *tra*, *tra-2* and *ix* are involved in the splicing of the primary *dsx* transcript in the female-specific mode, but also in the appropriate termination after the female-specific 3'exon. It is consistent with the genetic data to assume that the products of *tra* and *tra-2* are specifically

involved in splicing at the female-specific 3'exon. In a cell that lacks the products of *tra* or *tra-2*, the male-specific splice site is recognized, and the individual will display the male phenotype.

The gene products of *tra* and *tra-2* could be part of a complex known as spliceosome (Maniatis and Reed, 1987; Sharp, 1987). It is likely that different introns are recognized by spliceosomes consisting of different sets of proteins. Only the splicing of the female-specific exon of the *dsx* gene needs the determining factors of *tra* and *tra-2*.

Individuals (XX) that are homozygous for *ix* are transformed into intersexes. The function of the *ix* product therefore could be to terminate transcription (Birnstiel et al., 1985) of *dsx* after the female-specific 3'exon. In XX cells that lack functional *ix* products, the *dsx* transcript could be spliced in the female way at the fourth, female-specific exon, but without termination also the splice site of the male specific exon would be used, and the resulting messenger or the translated protein will neither function as F nor as M. The phenotype of the individual will be intersexual corresponding to a non-functional *dsx* product.

The *tra* and *tra-2* mutations behave like homeotic mutations and the presence or absence of functional products of the wild-type genes are essential to establish and maintain one of two possible pathways. The proposed regulatory mechanism of their action would form a new way to achieve a developmental switch, different from what is known for typical homeotic genes which regulate the expression of other genes by selective DNA binding (Gehring, 1985; Gehring and Hiromi, 1986; Porter and Smith, 1986; Gehring, 1987).

4.7. Function of the male-specific *tra* transcript

We did not expect the appearance of a male-specific transcript at the *tra* locus and we do not see a function for a *tra* product in males with our methods of detection. Therefore, we consider that the male-specific RNA is transcribed constitutively from a different promoter than the 0.9kb female-specific transcripts. It is possible that the 1.6kb female-specific transcript derives from the same promoter but is differently processed. Another explanation would be that the male-specific *tra* transcript functions to repress transcription of the female-specific RNA. Presence of male-specific transcripts was also reported for the gene *Sxl* by Maine et al. (1985a; 1985b), who speculated that the male-specific transcripts of *Sxl* repress transcription of the female-specific RNAs.

4.8. Evolutionary outlook on sex-determination

Sex-determination, as it works in *Drosophila melanogaster*, is certainly only one mechanism to establish a bipolar developmental system. Starting with the sex-transforming mutation *transformer* which seems to hold a key position in sexual development we wanted to investigate the way in which it exerts its control.

The DNA sequence of the *tra* gene gives us a tool, by looking for homologous genes in other species, to investigate how conserved are the sex-determining regulatory genes of within a systematic group. We expect to find genes with homologous functions in sex-determination in other *Drosophila* species, but probably also in related insects. It will be interesting to learn about the age of the sex-determining genes in *Drosophila* by calculating the evolutionary distance of a conserved *tra* gene in different related species.

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REFERENCES

- Aviv, H. and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* 69, 1408-1412.
- Baker, B. and Belote, J., (1983). Sex determination and dosage compensation in *Drosophila melanogaster*. *Annu. Rev. Genet.* 17, 345-393.
- Baker, B. and Ridge, K., (1980). Sex and the single cell. 1. On the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* 39, 383-423.
- Baker, B., Nagoshi, R. and Burtis, K., (1987). Molecular genetic aspects of sex-determination in *Drosophila*. *Bio Essays* 6, 66-70.
- Belote, J. and Baker, B., (1982). Sex determination in *Drosophila melanogaster*: analysis of transformer-2, a sex determining locus. *Proc. Natl. Acad. Sci. USA* 79, 1568-1572.
- Belote, J., Handler, A., Wolfner, M., Livac, K. and Baker, B., (1985a). Sex-specific regulation of yolk protein gene expression in *Drosophila*. *Cell* 40, 339-348.
- Belote, J., McKeown, M., Andrew, D., Scott, T., Wolfner, M. and Baker, B., (1985b). Control of sexual differentiation in *Drosophila melanogaster*. *Cold Spring Harbor Symp. Quant. Biol.* 50, 606-614.
- Birnstiel, M.L., Busslinger, M. and Strub, K., (1985). Transcription termination and 3' processing: The end is in site! *Cell* 41, 349-359.

- Bossy,B., Hall,L.M.C. and Spierer,P., (1984). Genetic activity along 315kb of the *Drosophila* chromosome. *EMBO J.* 3, 2537-2541.
- Bownes,M. and Nöthiger,R., (1981). Sex-determining genes and vitellogenin synthesis in *Drosophila melanogaster*. *Mol. Gen. Genet.* 182, 222-228.
- Bridges,B., (1921). Triploid intersexes in *Drosophila melanogaster*. *Science* 54, 252-254.
- Bridges,B., (1925). Sex in relation to chromosomes and genes. *Amer. Nat.* 59, 127-137.
- Bryant,P.J., (1978). Pattern formation in imaginal discs. The genetics and biology of *Drosophila* Vol. 2, p230-265. (Edited by Ashburner,M. and Wright,T., Academic Press London.
- Butler,B., Pirrotta,V., Irminger-Finger,I. and Nöthiger,R. (1986). The sex-determining gene *tra* of *Drosophila*: molecular cloning and transformation studies. *EMBO J.* 5, 3607-3613.
- Casanova,J., Sanchez-Herrero,E. and Morata,G., (1986). Identification and characterization of a parasegment-specific regulatory element of the *Abdominal-B* gene of *Drosophila*. *Cell* 47, 627-636.
- Chirgwin,J.M., (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294-5298.
- Cline,T., (1978). Two closely linked mutations in *Drosophila melanogaster* that are lethal to opposite sexes and interact with *daughterless*. *Genetics* 90, 683-698.
- Cline,T., (1979). A male-specific mutation in *Drosophila melanogaster* that transforms sex. *Dev. Biol.* 72, 266-275.

Cline,T., (1983). The interaction between *daughterless* and *sex-lethal* in triploids: A novel sex-transforming maternal effect linking sex-determination and dosage compensation in *Drosophila melanogaster*. Dev. Biol. 95, 260-274.

Cline,T., (1984). Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state. Genetics 107, 231-277.

Cline,T., (1985). Primary events in the determination of sex in *Drosophila melanogaster*. In Origin and Evolution of Sex (ed.H.O.Halvorson and A.Monroy), pp.301-327. Alan R.Liss, New York.

Cline,T., (1986). A female-specific lethal lesion in an X-linked positive regulator of the *Drosophila* sex determination gene, *Sex-lethal*. Genetics 113, 641-663.

Ehrensperger,P., (1983). Die Entwicklung der bisexuellen Anlage der Genitalien und Analien, untersucht an verschiedenen Geschlechtsmutanten der Taufliege *Drosophila melanogaster*. Mitt. Aarg. Naturf. Ges., 30, 145-235.

Epper,F. and Bryant,P., (1983). Sex-specific control of growth and differentiation in the *Drosophila* genital disc, studied using a temperature-sensitive *transformer-2* mutation. Dev. Biol. 100, 294-307.

Epper,F. and Nöthiger,R., (1982). Genetic and developmental evidence for a repressed genital primordium in *Drosophila melanogaster*. Dev. Biol. 94, 163-175.

Fjose,A., McGinnis,W. and Gehring,W., (1985). Isolation of a homeobox-containing gene from the *engrailed* region of *Drosophila* and the spatial distribution of its transcripts. Nature 313, 284-289.

Gehring,W.J., (1985). The Homeo Box: A key to the understanding of Development? Cell 40, 3-5.

Gehring,W.J., (1987). Homeotic genes, the *homeobox*, and the spatial organization of the embryo. The Harvey Lectures 81, 153-172.

Gehring,W. and Hiromi,Y., (1986). Homeotic genes and the homeobox. Ann Rev. Genet. 20, 147-173.

Hafen,E., Levine,M. and Gehring,W., (1983). An improved *in situ* hybridization method for the detection of cellular RNAs in *Drosophila* tissue sections and its application for localizing transcripts of the homeotic *Antennapedia* gene complex. EMBO J. 2, 617-623.

Hanahan,D., (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557-568.

Hodgkin,J., (1985). Males, hermaphrodites and females: sex determination in *Caenorhabditis elegans*. Trends in Genetics 1, 85-88.

Laughon,A. and Scott,M.P., (1984). Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA binding proteins. Nature 310, 25-30.

Legerski,R.J., Hodnett,J.L. and Gray,H.B.J., (1978). Extracellular nucleases of *Pseudomonas* Bal31III. Use of the double-strand deoxyriboexonuclease activity as the basis of a convenient method for the mapping of fragments of DNA produced by cleavage with restriction enzymes. Nucl. Acids Res. 5, 1445-1452.

Lewis,E.B., (1978). A gene complex controlling segmentation in *Drosophila*. Nature 276, 565-560.

Lindsley,D.L. and Grell,E.H., (1968). Genetic variations of *Drosophila melanogaster*. Carnegie institution of Washington, Publ. 627.

Lucchesi,J. and Manning,G., (1987). Genetics of dosage compensation in *Drosophila melanogaster*. Adv. in Genetics, (in press).

Lucchesi,J. and Skripsky,J., (1981). The link between dosage compensation and sex-determination in *Drosophila melanogaster*. Chromosoma 82, 217-227.

Maine,E.M., Salz,H., Schedl,P. and Cline, T., (1985a). *Sex-lethal*, a link between sex determination and sexual differentiation in *Drosophila melanogaster*. Cold Spring Harbor Symp. Quant. Biol. 50, 595-604.

Maine,E.M., Salz,H.K., Cline,T. and Schedl,P., (1985b). The *Sex-lethal* gene of *Drosophila*: DNA alterations associated with sex-specific lethal mutations. Cell, 43, 521-529.

Maniatis,T. and Reed,R., (1987). The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature 325, 673-678.

McGinnis,W., Garber,R., Wirz,J.,Kuroiwa,A. and Gehring,W.J., (1984). A homologous protein coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. Cell 37, 403-408.

McKeown,M., Belote,J. and Baker,B., (1987). A Molecular Analysis of *transformer*, a gene in *Drosophila* that controls female sexual differentiation. Cell, 48, 498- 499.

Morata,G. and Lawrence,P.A., (1977). Homeotic genes, compartments and cell determination in *Drosophila*. Nature 265, 211-216.

Mount,S.M., (1982). A catalogue of splice junction sequences. Nucl. Acids Res. 10, 459-472.

Nasmyth,K.A., (1982). Molecular genetics of yeast mating type. Annu. Rev. Genet. 16, 439-500.

Nöthiger,R., Leuthold,M., Andersen,N., Gerschwiler,P., Grüter,A., Keller,W., Leist,C., Roost,M. and Schmid,H., (1987). Genetic and developmental analysis of the sex-determining gene *doublesex (dsx)* of *Drosophila melanogaster*. Genetics (in press).

Nöthiger,R. and Steinmann-Zwicky,M., (1985). Sex determination in *Drosophila*. Trends in Genetics, 1, 209-215.

Okayama,H. and Berg,P., (1982). High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. 2, 161-169.

Poole,S., Kauvar,L., Drees,B. and Kornberg,T., (1985). The *engrailed* locus of *Drosophila*: Structural analysis of an embryonic transcript. Cell 40, 37-43.

Porter,S.D. and Smith,M., (1986). Homeo-domain homology in yeast MAT α 2 is essential for repressor activity. Nature, 320, 766-768.

Proudfoot,N.J. and Brownlee,G.G., (1976). 3'-non-coding region sequences in eukaryotic mRNA. Nature 263, 211-214.

Rubin,G. and Spradling,A., (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science 218, 348-353.

Sanchez,L. and Nöthiger,R., (1982). Clonal analysis of *Sex-lethal*, a gene needed for female sexual development. Wilhelm Roux's Arch. Dev. Biol. 191, 211-217.

Sanchez,L. and Nöthiger,R., (1983). Sex determination and dosage compensation in *Drosophila melanogaster* : production of male clones in XX-females. EMBO J. 2, 485-491.

Sanger,F., Nicklen,S. and Coulson,A.R., (1977). DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Sanger,F., Coulson,A.R., Barrel,B.G., Smith,A.J.H. and Roe,B.A., (1980). Cloning of single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143, 161-178.

Schüpbach,T., (1985). A proper X-chromosome-autosome ratio and expression of the *Sex-lethal* locus are necessary for normal differentiation of female germ cells in *Drosophila melanogaster*. Genetics 109, 529-548.

Scott,M.P. and Weiner,A.J., (1984). Structural relationships among genes that control development: sequence homology between the Antennapedia, Ultrabithorax and fushi tarazu loci of *Drosophila*. Proc. Natl. Acad. Sci. USA 81, 4115-4119.

Sharp,P., (1987). Splicing of messenger RNA precursors. Science, 235, 766-771.

Southern,E., (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-511.

Spradling,A. and Rubin,G., (1982). Transposition of cloned P-elements into *Drosophila* germline chromosomes. Science 218, 341-347

Staden,R., (1980). Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nuc. Acids Res. 8, 3673-3694.

Staden,R. (1984a). Computer methods to locate signals in nucleic acids. Nucl. Acids Res. 12, 505-519.

Staden,R., (1984b). Measurements of the effects that coding of a protein has on a DNA sequence and their use for finding genes. Nucl. Acids Res., 521-538.

Steinmann-Zwicky,M. and Nöthiger,R., (1985a). A small region on the X-chromosome of *Drosophila* regulates a key gene that controls sex determination and dosage compensation. Cell 42, 877-887.

- Steinmann-Zwicky,M. and Nöthiger,R., (1985b). The hierarchical relation between X-chromosomes and autosomal sex-determining genes in *Drosophila*. EMBO J. 4, 163-166.
- Steller,H. and Pirrotta,V., (1985). A transposable P vector that confers selectable G418 resistance to *Drosophila* larvae. EMBO J. 4, 167-171.
- Sturtevant,A.H., (1945). A gene in *Drosophila melanogaster* that transforms females into males. Genetics 30, 297-299.
- Tompkins,L., (1986). Genetic Control of Sexual Behavior in *Drosophila melanogaster*. Trends in Genetics 2, 14-20.
- Watanabe,T.K., (1975). A new sex-transforming gene on the second chromosome of *Drosophila melanogaster* Jpn. J. Genet. 50, 269-271.
- White,R.A. and Wilcox,M., (1984). Protein products of the bithorax complex in *Drosophila*. Cell 39, 163-171.
- Wieschaus,E. and Nöthiger,R., (1982). The role of the *transformer* genes in the development of the genitalia and analia of *Drosophila melanogaster*. Dev. Biol. 90, 320-334.
- Yanisch-Perron,C., Vieira,J. and Messing,J., (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

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